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WATER DRIVE STUDIES ON HYPOPHYSECTOMIZED EFTS OF DIEMYCTYLUS VIRIDESCENS. PART I. THE ROLE OF THE LACTOGENIC HORMONE

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It is well known that following metamorphosis the eastern, spotted newt *Diemyctylus viridescens* passes into a terrestrial or red eft stage which lasts from three to four years before the animals migrate to water where they become sexually mature. In certain parts of Long Island and in the Woods Hole area, however, Noble (1926, 1929) found that the eft stage failed to develop. There has been considerable interest in the role played by the endocrine glands in the events associated with the migration of efts from land to water. Adams (1932) was able to induce adult skin texture and pigmentation in efts injected with an anterior lobe preparation (phyone), while Dawson (1936) showed that pituitary preparations administered to efts brought about the maturation of the lateral line system. The studies of Reinke and Chadwick (1939) demonstrated that efts receiving implants of whole adult pituitaries or anterior lobes voluntarily migrated to water from 2 to 6 days following treatment. The test animals acquired a smooth, moist skin and showed a tendency toward the olive pigmentation of the adult. In certain cases keeling of the tail was evident after an extended period. The thyroids showed some stimulation as the result of the implants but the gonads remained unaffected. Molting usually occurred on the second to fourth day following implantation. Gonadectomized efts molted and entered water from 4 to 8 days after implantation of adult pituitaries according to Reinke and Chadwick (1940). Thyroidectomized individuals and animals which had been both thyroidectomized and gonadectomized were forced to water following similar treatment, although in these cases molting was abnormal with pieces of cornified epithelium sloughing off in patches after the animals had assumed the aquatic habitat.

The failure of thyroidectomized efts to undergo a normal molt is understandable in the light of investigation by Adams and her co-workers. Adams *et al.* (1932) and Adams and Grierson (1932) have shown that a pituitary-thyroid relationship is necessary for proper molting. Changes in cutaneous circulation, rather than the stimulation of secretion of the cutaneous glands, may be of major importance to the molting process. According to Chadwick (1948), however, the thyroid exerts a direct effect on molting by the stimulation of the inter-papillary skin glands. An increased incidence in molting, noted by Chadwick and Jackson (1948) following

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the injection of efts with prolactin, may have been due to the stimulation of cell division in the epidermis.

Chadwick (1940a) induced water drive in efts ranging from 60 to 95 mm. in length with injections of Antuitrin G (Parke Davis). This preparation was less effective than implants of adult newt pituitaries, as it failed to induce water drive in smaller animals or those which had been thyroidectomized. Prolactin was identified with the active water drive principle of the anterior lobe by Chadwick (1940b). Injections of 14 to 20 mg. of prolactin (Eli Lilly unidentified lot) caused water drive in all normal, thyroidectomized and gonadectomized efts within a period of 10 days. Chadwick (1940c, 1941) obtained the migration of efts to water following intramuscular and intraperitoneal implants of hypophyses of a variety of vertebrates such as the water snake (Natrix), the domestic fowl and several genera of urodele and anuran amphibians. That the water drive reaction may be more complex is indicated by Dr. Richard W. Payne (unpublished data) who obtained positive results after the injection of a wide number of pituitary preparations, several of which showed negative prolactin activity by the pigeon crop assay. Tuchmann-Duplessis (1948, 1949) has shown that the administration of 60–120 Riddle-Bates units of prolactin to the land stage of *Triturus cristatus* and *T. alpestris* resulted in the migration of the animals to water and the assumption of pigmentation and morphological characters associated with the aquatic, reproductive phase. The cloaca became enlarged while the gonads and prostate became active. Prolactin administered to castrated males produced only water drive and color changes.

The results reported above indicate that prolactin is probably the active principle concerned with the initiation of water drive and that the thyroid, while not necessary to this reaction, facilitates the process by conditioning normal molting. Nevertheless, the situation remains confused, considering that a number of hormone preparations other than prolactin have produced water drive activity, and it is not clear which of the various phenomena accompanying migration (pigment changes, etc.) are stimulated directly by the water drive factor and which result from endogenous release of other endocrine substances through stimulation of the pituitary. The present investigation is part of an extended study seeking to clarify the complex situation involved in the transformation of the terrestrial eft to the aquatic adult. The use of hypophysectomized test animals has been necessary in order to rule out the endogenous release of prolactin itself by a specific testing agent or nonspecific "shock" effect and to eliminate synergic reaction within the pituitary. Grant and Grant (1956) have previously indicated that prolactin causes water migration and skin changes in hypophysectomized efts but none of the other changes toward the adult condition.

"Water drive" is used below to indicate the actual migration of efts to water and "water drive syndrome" to designate migration plus associated morphological changes, etc. The term "drive" is used loosely and is not necessarily meant to imply the operation of precise directive factors.

The authors wish to express their indebtedness to Dr. Grace E. Pickford, Bingham Oceanographic Laboratory, Yale University, for suggesting the project and for furnishing many of the preparations used. We are further indebted to

Dr. C. H. Li of the Hormone Research Laboratory, University of California, for the donation of a quantity of his highly purified prolactin used in the tests. This investigation was sponsored, in part, by a grant from the Mearl Corporation of New York City, through the kindness of Mr. Harry E. Mattin and Dr. Leon M. Greenstein, while a generous Grant-in-Aid from the Signa Xi-RESA Research Fund helped finance investigations through the past year. Our thanks are extended to Williams College and to Dr. S. A. Matthews, Chairman of the Department of Biology, for laboratory space and numerous facilities used during this investigation.

Materials and Methods

Efts were collected near Lyme Center, New Hampshire; Quechee, New York; Honesdale, Pennsylvania, and Williamstown, Massachusetts. Animals from different localities were segregated and kept in plastic boxes, 15–20 animals per box, on a thick bed of moss (usually Polytrichum). Room temperature ranged from 21°–24° C. Illumination was that of the room except for a few hours each day when the containers were subjected to direct illumination from an overhead lamp. This procedure aided the growth of the moss, the presence of which seems to be extremely helpful in keeping animals healthy. Attempts made to keep efts on a floor of wet, cellulose sponge proved quite unsuccessful. Animals were fed Enchytraeus worms and blowfly larvae. Some test animals were kept without feeding for extended periods in a refrigerator at approximately 5° C. Hypophysectomized efts are particularly susceptible to infection, but 1% solutions of potassium bichromate or malachite green diluted 1:15,000 have proved efficient prophylactic agents.

The total length of experimental animals ranged from 40 to 74 mm. with weight varying from 0.5 to 1.4 gm. The probable age of such animals was from 1 to 2.5 years and all were well removed from the naturally occurring aquatic phase of their cycle. Snout-vent measurements, which have proved an accurate standard in herpetological work, were also recorded.

Animals were anesthetized in a 0.5% solution of chloretone and hypophysectomized with the aid of a suction pipette attached to an aspiration unit. At the termination of the experiments hypophysectomy was verified histologically on most specimens. Two weeks after hypophysectomy subcutaneous injections were made with a 27-gauge, Huber point needle. In general, injections were made every other day at various concentrations of solution, but a constant volume of 0.1 cc. at each injection was maintained. Water drive responses were studied in containers possessing equal areas of land and water. The time taken for the assumption of the aquatic habitat, the number of molts and the duration of the aquatic phase of life were recorded for all individuals as far as possible.

The various preparations used in the injections were made up in a standard amphibian Ringer's, with controls receiving the same volume of saline as experimental animals. The following pituitary preparations were tested: FSH (swine, Armour Lot No. K45208R), LH (sheep, Armour Lot 227–80), TSH (Armour Lot 317–51), Antuitrin S (Parke Davis Lot P459D), ACTH (hog, Armour Lot K 52204), posterior pituitary preparation (hog, Lot 503), GH (beef growth hormone, Wilhelmi Lot B168), prolactin (Sheep, Schering Lot 4g Hyex 4, Armour sheep Lot 759–CCC and the highly purified sheep preparation of Li), MSH

TABLE I

Results of water drive studies following the treatment of land phase Diemyctylus viridescens with various pituitary preparations

Treatment	Hypophysectomy	Wt., gm.	Length, mm. Total/Standard	Total dose, mg.	Results	Molting
*FSH Armour**	−	0.55	55/26	8	9 days to water	+
	−	0.80	55/29	8	10 days to water	+
	+	1.20	67/36	8	partial response	abnormal
	+	1.40	69/36	8	8 days to water	abnormal
	+	0.85	67/34	0.8	negative response	−
	+	0.72	63/32	0.8	negative response	−
	+	0.47	56/29	0.8	negative response	−
LH Armour	−	0.81	56/33	8	negative response	−
	−	0.47	48/23	8	negative response	−
	+	1.10	73/36	8	negative response	−
	+	1.12	68/37	8	negative response	−
GH Wilhelmi	+	1.10	73/38	0.8	negative response	+
	+	0.53	60/29	0.8	negative response	−
	+	0.53	59/32	0.8	negative response	−
	+	0.72	63/33	0.8	negative response	−
	+	0.74	63/32	0.8	negative response	−
	+	0.53	51/31	0.8	negative response	−
ACTH Armour	+	0.62	57/26	0.8	negative response	−
	+	0.71	64/32	0.8	negative response	+
	+	0.65	59/32	0.8	negative response	−
	+	0.67	62/34	0.8	negative response	−
	+	0.68	64/33	0.8	negative response	−
A corticotropin (Li)	+	0.84	65/35	0.8	negative response	−
Posterior pituitary Armour	+	1.22	72/37	0.8	negative response	−
	+	0.68	58/32	0.8	negative response	−
	+	0.50	52/31	0.8	negative response	−
	+	0.62	54/31	0.8	negative response	−
TSH Armour	+	0.61	58/32	0.8	negative response	−
	+	0.74	61/34	0.8	negative response	+
	+	0.84	65/35	0.8	negative response	+
	+	0.57	54/33	0.8	negative response	+
	+	0.62	60/33	0.8	negative response	+
Antuitrin S Parke & Davis	−	0.65	52/30	6	dead 7th day	−
	−	0.72	53/35	4	dead 5th day	−
MSH Armour	+	0.88	66/35	2	negative response	−
	+	0.47	51/28	2	negative response	−
	+	0.55	55/30	2	negative response	−
Intermedin (Li)	+	0.60	58/39	0.8	negative response	−

* All animals receiving 8 mg. of FSH showed a tendency toward the olive pigmentation of the adult.

(melanophore stimulating hormone, Armour Lot R 527225). According to Steelman *et al.* (1953), the assay for the FSH preparation shows it to be contaminated with 0.5 I.U. of prolactin per mg., a fact which is of importance in interpreting the results given below.

Results

(*a*) *Various mammalian pituitary preparations*

The detailed results of this series of injections are given in Table I. LH, TSH, ACTH, MSH, posterior pituitary, GH and Antuitrin S failed to induce water drive in any of the animals tested. One animal given 0.8 mg. of ACTH-free Intermedin (Li) also gave a negative response. Most efts treated with TSH underwent a normal molt following injections, while one eft of the GH series and one of the ACTH series showed this reaction. All other preparations failed to produce normal molts in hypophysectomized individuals with the result that the

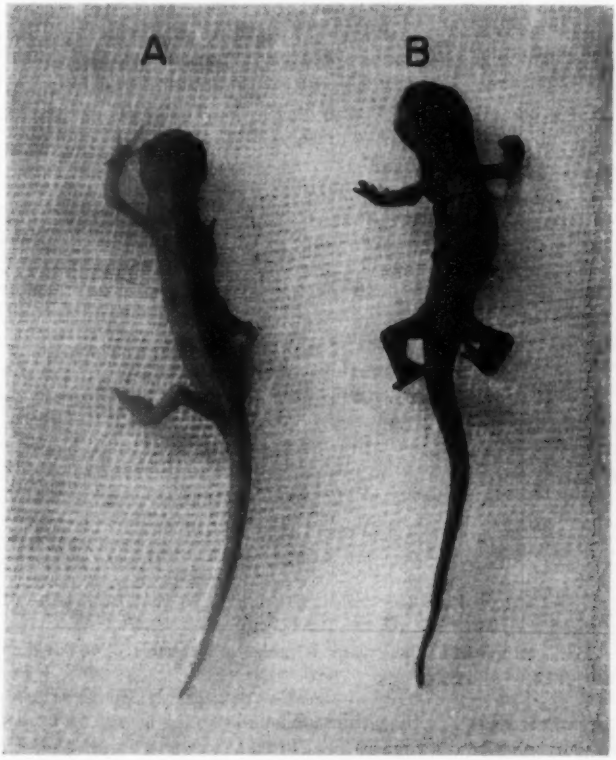

FIGURE 1. A normal eft (A) is shown beside a hypophysectomized animal (B), which having failed to molt is covered with a thick layer of cornified epithelium.

TABLE II

Results of water drive studies following the treatment of land phase Diemyctylus viridescens with prolactin

Treatment	Wt., gm.	Length, mm. Total/Standard	Total dose, mg.	Effective dose I.U.*	Results	Molting
Non-hypophysectomized *Schering prolactin* 30 I.U./mg.	0.54	50/28	8	240	7 days to water	+
	0.64	51/29	8	240	7 days to water keeling of tail and olive pigmentation	+
Hypophysectomized *Armour prolactin* 25–30 I.U./mg.	1.12	74/39	8	216	8 days to water	abnormal
	0.41	56/29	8	162	6 days to water	–
	0.61	63/33	0.8	23	10 days to water	abnormal
	0.43	55/28	0.8	23	8 days to water	–
	0.76	57/31	0.8	23	7 days to water	abnormal
	0.80	60/29	0.08	—	partial response	abnormal
Hypophysectomized *Prolactin (C.H.Li)* 35 I.U./mg.	0.66	57/32	0.4	14	10 days to water	–
	1.14	69/36	0.4	10.5	5 days to water	–
	0.63	59/32	0.4	10.5	5 days to water	abnormal
	0.75	65/34	0.4	7	4 days to water	abnormal
	0.59	58/32	0.4	10.5	5 days to water	–
	0.48	55/29	0.4	—	Dead on 3rd day	
	0.73	60/32	0.4	—	Dead on 4th day	
	0.80	62/34	0.4	14	7 days to water	–
	0.71	57/31	0.4	14	8 days to water	abnormal
	0.35	40/26	0.4	14	7 days to water	abnormal
	0.60	57/30	0.4	14	7 days to water	–
	0.61	54/29	0.4	14	8 days to water	abnormal
	0.97	69/36	0.4	10.5	6 days to water	abnormal
Hypophysectomized *Prolactin (C.H.Li)* 35 I.U./mg.	0.87	58/34	0.04	1.4	8 days to water	–
	0.92	64/35	0.04	1.4	10 days to water	–
	0.63	57/29	0.04	1.4	8 days to water	abnormal
	0.82	65/34	0.04	1.4	8 days to water	–
	0.44	50/27	0.04	1.4	8 days to water	abnormal
	0.78	70/35	0.04	1.05	6 days to water	–

* Effective dose is estimated as the amount of prolactin efts had received at the time of their assumption of an aquatic habitat.

efts rapidly became covered with a thick, black layer of cornified epithelium until even the eyes were obscured (Fig. 1).

Both normal and hypophysectomized efts receiving a total of 8 mg. of FSH showed water drive activity from 8 to 10 days following the initial injections. One

animal failed to give a complete reaction and was in and out of water for several weeks before returning permanently to land. In these animals molting was abnormal with the skin sloughing off in irregular patches after the efts had entered water. There was a trend in the pigmentation of all individuals toward the adult olive, though this was more marked in the non-operated efts. It is interesting that tests for water drive in animals receiving 0.8 mg. of FSH were completely negative, and that molting failed to occur.

(*b*) *Tests with prolactin*

Two unhypophysectomized animals receiving 8 mg. (240 I.U.) of Schering prolactin migrated to water on the seventh day following the initial injections and within a few weeks had acquired many features associated with the water drive syndrome (*i.e.,* smooth, moist skin, olive pigmentation and keeling of the tail). Other prolactin preparations were injected into hypophysectomized efts in doses varying from 8.0 to 0.04 mg. as shown in Table II. In all cases where death did not occur before the injections were completed, the tests were positive. The animals assumed the aquatic habitat from 4 to 10 days following the first injection. It should be noted that several animals migrated before all injections had been completed and it is therefore desirable to give results in terms of the effective dose (*i.e.,* the dose animals had received at the time of the water-drive response) rather than total dose. The range in effective dose was from 216 to 1.05 I.U.

The water drive reaction is very positive as animals giving the reaction remain completely submerged, take food under water and will immediately return to water if placed on land. One eft receiving 0.08 mg. (2.3 I.U.) failed to give the complete reaction but migrated alternately between land and water over the time observed. Records on the duration of water drive are far from complete as most test animals died before leaving water. However, in a number of cases animals actually did return to land after periods varying from two to five weeks.

It is of particular significance to note that whereas all hypophysectomized animals showed positive water drive in response to treatment with prolactin, they failed to assume the olive pigmentation and tail keel associated with the water drive syndrome. When molting occurred it was abnormal, but beneath the patches of thickened corneum the smooth, moist skin retained the orange pigmentation of the eft and showed no tendency toward the adult olive. No keeling of the tail was apparent in any individual even after extended periods in water.

Conclusions

The primary concern of the present investigation was to determine as precisely as possible the endocrine factor responsible for water drive in the red eft. From the results reported above we are in agreement with Chadwick (1940c) that prolactin is the active principle. All animals treated with this substance migrated to water and assumed a smooth skin texture similar to that of the adult. As tests were conducted on hypophysectomized efts the possibility of hypophyseal synergy or endogenous release must be ruled out. All other pituitary preparations administered gave a negative reaction with the exception of the 8-mg. dose of FSH. This is understandable, as the assay for the gonadotropin shows it to be contaminated with

lactogenic hormone. Both the Armour prolactin and the homogeneous preparation of Li produced positive results in animals receiving as little as 2.3 to 1.4 I.U. per effective dose. The 4 I.U. of prolactin contained in the FSH were therefore quite sufficient to initiate water drive. No minimum dosage level for the water drive reaction has yet been established but the failure of 0.8 mg. FSH to elicit positive results may indicate it to be about 0.4 I.U. Though there was some variability in the time animals responded to treatment with prolactin, there is at present no indication of a dose-response relationship and it is suggested that the reaction may follow the all-or-none principle.

Reinke and Chadwick (1940) have shown that the thyroid and gonads are not directly involved in the water drive response and initial histological survey of our prolactin tests shows no thyrotropic or gonadotropic activity. The lactogenic hormone is not effective in promoting molt in hypophysectomized animals as it was in normal efts studied by Chadwick and Jackson (1948). However, as molting did occur in efts receiving injections of TSH it suggests that the increased molting reported by Chadwick and Jackson (1948) in intact animals was due to the endogenous release of TSH resulting from treatment.

Though our investigations are in general agreement with those of Chadwick, we cannot support his assumption that prolactin effects the entire water drive syndrome. Work on hypophysectomized animals indicates that the problem is considerably more complex and can tentatively be divided into four major steps.

1. Migration to water and change of skin texture: induced by prolactin.
2. Normal molting which facilitates but does not directly affect water drive: release of thyroid hormone mediated through the pituitary (TSH).
3. Appearance of olive pigmentation: unknown principle involved, possibly MSH.
4. Morphological characteristics associated with water drive such as keeling of the tail and development of the lateral line system: unknown principle or principles involved.

It is tempting to suggest that prolactin may initiate the entire water drive syndrome by triggering the endogenous release of other endocrines which induce many changes associated with the aquatic phase. The identification of these substances, the parts of the cycle they effect and possible interrelationships involved will be taken up in future papers. In conclusion it appears safe to say that the lactogenic hormone produces water drive and skin change, and that the red eft test for the presence of prolactin (1 I.U. or above) is a positive and reliable one.

Summary

1. Other investigators have shown that the land (eft) stage of *Diemyctylus viridescens* can be induced to enter water and assume adult pigmentation and morphological characteristics following treatment with various pituitary preparations. Hypophysectomized efts were used in the present experiment in order to assure positive identification of the active, water drive principle.

2. Operated animals treated with LH, growth hormone, ACTH, posterior pituitary preparation, TSH, Antuitrin S and melanophore-stimulating hormone gave a negative response. Eight-milligram injections of FSH produced water drive in

several animals, but this was most probably due to the contamination of the substance with prolactin.

3. Most hypophysectomized efts, with the exception of those receiving TSH, either failed to molt or underwent an abnormal molt after the animals had been induced to enter water.

4. Operated animals receiving injections of prolactin (240 to 1.05 I.U.) migrated to water from 4 to 10 days following treatment. However, they failed to acquire adult pigmentation and associated characteristics.

5. The lactogenic hormone has been identified as the principle which initiates the migration of efts to water and the water drive test for prolactin is considered to be reliable.

LITERATURE CITED

ADAMS, A. E., 1932. Observations on the effect of anterior pituitary extract (phyone) on the 'red phase' of *Triturus viridescens*. *Anat. Rec.*, **52**: 46. (Abst.)

ADAMS, A. E., AND M. C. GRIERSON, 1932. Cornification and molting in *Triturus*. *Proc. Soc. Exp. Biol. Med.*, **30**: 341–344.

ADAMS, A. E., A. KUDER AND L. RICHARDS, 1932. The endocrine glands and molting in *Triturus viridescens*. *J. Exp. Zool.*, **63**: 1–55.

CHADWICK, C. S., 1940a. Induction of water drive in *Triturus viridescens* with anterior pituitary extract. *Proc. Soc. Exp. Biol. Med.*, **43**: 509–511.

CHADWICK, C. S., 1940b. The water drive in *Triturus viridescens* as an effect of the growth promoting hormone of the anterior hypophysis. *J. Tenn. Acad. Sci.*, **15**: 412.

CHADWICK, C. S., 1940c. Identity of prolactin with water drive factor in *Triturus viridescens*. *Proc. Soc. Exp. Biol. Med.*, **45**: 335–337.

CHADWICK, C. S., 1941. Further observations on the water drive in *Triturus viridescens*. II. Induction of the water drive with the lactogenic hormone. *J. Exp. Zool.*, **86**: 175–187.

CHADWICK, C. S., 1948. Evidence of a thyroid-skin gland relationship in the induction of molting in the red eft of *Triturus viridescens*. *Anat. Rec.*, **101**: 678. (Abst.)

CHADWICK, C. S., AND H. R. JACKSON, 1948. Acceleration of skin growth and molting in the red eft of *Triturus viridescens* by means of prolactin injections. *Anat. Rec.*, **101**: 718. (Abst.)

DAWSON, A. B., 1936. Changes in the lateral line organs during the life of the newt *Triturus viridescens*. A consideration of the endocrine factors involved in the maintenance of differentiation. *J. Exp. Zool.*, **74**: 221–237.

GRANT, W. C., AND J. A. GRANT, 1956. The induction of water drive in the land stage of *Triturus viridescens* following hypophysectomy. *Anat. Rec.*, **125**: 604. (Abst.)

NOBLE, G. K., 1926. The Long Island newt; a contribution to the life history of *Triturus viridescens*. *Amer. Mus. Nov., No. 228.*

NOBLE, G. K., 1929. Further observations on the life-history of the newt *Triturus viridescens*. *Amer. Mus. Nov., No. 348.*

REINKE, E. E., AND C. S. CHADWICK, 1939. Inducing land stage of *Triturus viridescens* to assume water habitat by pituitary implants. *Proc. Soc. Exp. Biol. Med.*, **40**: 691–693.

REINKE, E. E., AND C. S. CHADWICK, 1940. The origin of water drive in *Triturus viridescens*. I. Induction of the water drive in thyroidectomized and gonadectomized land phases by pituitary implantations. *J. Exp. Zool.*, **83**: 224–233.

STEELMAN, S. L., W. A. LAMONT, W. A. DITTMAN AND E. J. HAWRYLEWICZ, 1953. Fractionation of the swine follicle stimulating hormone. *Proc. Soc. Exp. Biol. Med.*, **82**: 645–647.

TUCHMANN-DUPLESSIS, H., 1948. Développement des caractères sexuels du Triton traité par des hormones hypophysaires gonadtropes et lactogènes. *C. R. Soc. Biol.*, **142**: 629–630.

TUCHMANN-DUPLESSIS, H., 1949. Action de l'hormone gonadtrope et lactogène sur le comportment et les caractères sexuels secondaires du triton normal et castré. *Arch. Anat. Micr. Morph. Exp.*, **38**: 302–317.

THE SENSITIVITY OF ECHOLOCATION IN BATS

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The full significance of acoustic orientation in bats can only be understood when we know what kinds of objects are detected and at what distances. Is it true, as is often assumed, that echolocation is limited to very close ranges of a foot or two? To what extent can bats discriminate between different objects? Are they merely aware that something is or is not directly ahead, or does echolocation inform them about the distance, size, numbers, direction and speed of motion of whatever is returning the echoes? Insectivorous bats seem to use echolocation in the pursuit and capture of flying insects; do they distinguish between various kinds of insects? Some continue to hunt insects in the rain; how can they tell the beetles from the raindrops? It would also be helpful to know how the acuity of echolocation varies among the several groups of bats which employ quite different intensities and patterns of sound for echolocation (Möhres, 1953; Griffin and Novick, 1955; Griffin, 1958).

These and related questions call for a better understanding of the sensitivity and effective range of echolocation, and this paper describes some new measurements of the distances at which bats first react to the presence of small wires. Although the smaller species of bats often fly very close to large objects such as the walls of a room before showing any sign of awareness that something is ahead, they do change the pattern of their orientation sounds at somewhat greater distances. For example, a *Myotis lucifugus* commonly increases its pulse repetition rate from perhaps 5 to 10 per second before take-off to 15 or 20 per second during ordinary flight and to 50 or more per second when landing or dodging small obstacles. This increase is closely correlated with success in avoiding objects such as wires. The rate rises every time a normal or blindfolded bat approaches the wires, but deafened bats show no such increase as they fly up to wires which they cannot detect (Galambos and Griffin, 1942). We have utilized this characteristic increase in pulse repetition rate to determine the distance at which bats first react to obstacles of various sizes by thus changing the pattern of their emitted sounds, and the results demonstrate a greater range and sensitivity of echolocation than had previously been recognized.

We are happy to acknowledge our gratitude to the Office of Naval Research for the support of these studies through a contract with Harvard University. Reproduction in whole or in part is permitted for any purpose of the United States Government.

Methods

Bats were allowed to fly in the rectangular room shown in Figure 1. This room is 10 meters long, 3.7 meters wide, and 2.4 meters high; it was free from furniture or obstructions other than the wires, three observers, and a microphone and camera

mounted on tripods. Seven meters from the end of the room, marked A, was a row of vertical wires 30 cm. apart, and 5.5 meters from the same end, in an indentation in one wall, was an Auricon model CM-72 16 mm. sound motion picture camera with a 9.5 mm. lens. In all cases the flying bats stood out against the white background formed by the opposite wall, which was marked only by conspicuously numbered vertical stripes placed at 60-cm. intervals to provide a frame of reference. In each flight used in the present measurements, the bat was released close to point A and flew approximately straight towards the opposite wall, B, passing the wires 7 meters away from its starting point. Usually it turned in the remaining 3 meters or else landed on the end wall.

The observer who released the bat at point A noted its approximate distance from the wall opposite the camera as it flew towards and past the wires. A second observer turned the camera to follow the bat during its flight, which in a typical

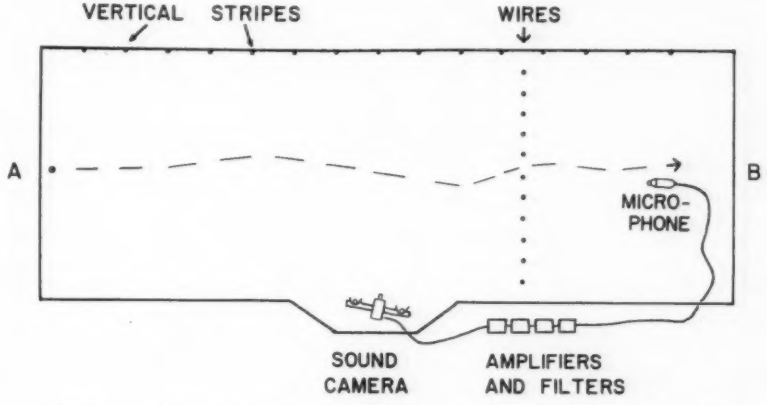

FIGURE 1. Diagram of room used for measurements of the distance of vocal reaction to the wires.

case approximated the dashed line of Figure 1. The third observer kept the microphone pointed towards the flying bat. The motion pictures showed the flight path of the bat as it appeared from the position of the camera silhouetted against the opposite wall, but a parallax correction (based on the first observer's notes of the bat's distance from the white wall) was necessary except when the animal was directly opposite the camera. The camera was so placed that any errors introduced by parallax were minimized in the region where the pulse repetition rate was beginning to change.

The high frequency sounds emitted by the bat as it flew the length of the room were picked up by a 640AA Western Electric condenser microphone placed 2.1 meters beyond the wires. The amplifiers and associated apparatus were similar to those used in previous studies of bat sounds (Griffin, 1950, 1953). The frequency band from 30 to 80 kc was selected by Spencer-Kennedy Laboratories model 301 and 302 variable electronic filters, 54 db/octave slope at 30 kc high pass and 18 db/octave slope at 80 kc low pass. The amplified signal was then passed through

a detecting circuit (the pulse detector used by Griffin and Novick, 1955), and the resulting clicks were recorded directly on the sound track of the same film containing the photographs. The developed film was studied with a time-motion study projector, the single frames being projected one at a time while the corresponding portion of the sound track was moved past a celluloid time scale calibrated in milliseconds. It was thus possible to measure directly for every pulse the position of the bat and the elapsed time since the last pulse.

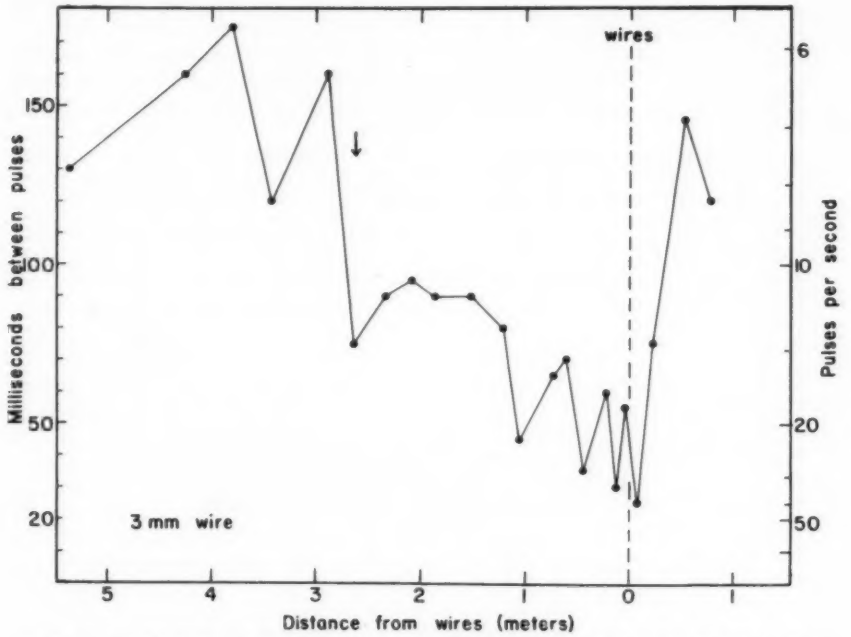

FIGURE 2. Variation of the pulse-to-pulse interval during one flight of a *Myotis lucifugus* through a row of 3-mm. wires along approximately the flight path shown in Figure 1. The arrow indicates the distance of the first vocal reaction.

The bats used in these experiments were *Myotis lucifugus* which had been in captivity for no more than one day, and all were in excellent condition. Six sizes of wire were used: 3 mm., 1.07 mm., 0.65 mm., 0.46 mm., 0.28 mm. and 0.18 mm. in diameter. The 3-mm. wire was rubber-covered, but all the others were bare iron or copper. Out of about 650 flights photographed, 146 were selected for analysis because the bats did react to the wires as demonstrated by straight flights through the wires, usually with a clear effort to dodge them. For this reason there was a larger proportion of misses than would otherwise have been the case. Flights with appreciable turns and flights near the walls, ceiling, or floor were excluded since a close approach to any object is likely to involve a change in pulse repetition rate. We also excluded flights in which the record of the pulses on the sound track was

of low amplitude or was complicated by noise, so that there was a danger that some of the pulses might be overlooked in studying the record. Other flights were excluded because the pulse repetition rate varied widely during the 3 to 4 meters of straight flight from point A to the vicinity of the wires or did not return to approximately the same level after the bat passed through the wires. Nor were any flights used unless we were confident that the photographs established the bat's position with an accuracy of ± 10–15 cm. over at least the major part of its flight through the wires. The time required for sound to travel from bat to microphone was only

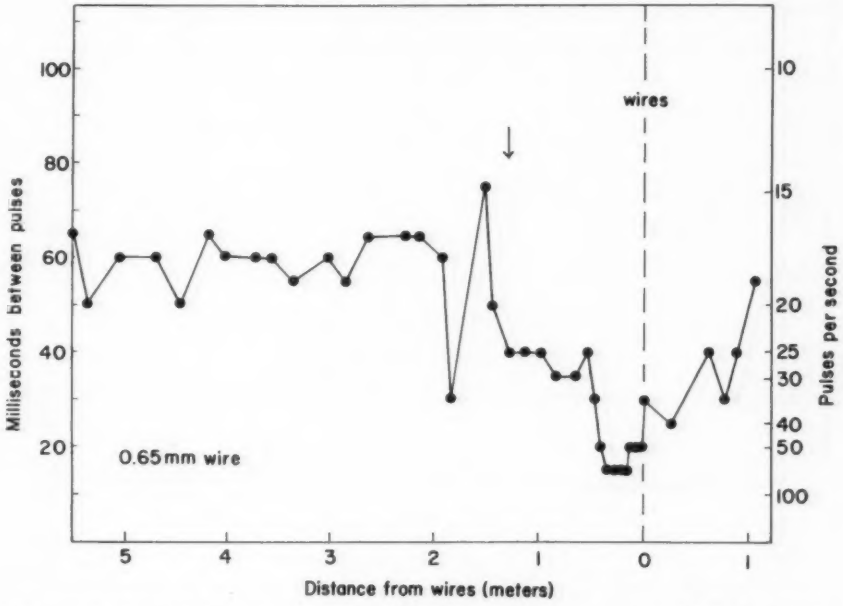

FIGURE 3. Intervals between pulses emitted by a bat flying through a row of 0.65-mm. wires.

about 0.03 second at the very most, and it decreased gradually as the animal flew towards the wires. Hence the acoustic delay had no appreciable effect on the measurement of the interval between pulses.

RESULTS

More than 500 flights through the wires showed the characteristic increase in pulse repetition rate with only two or three exceptions, all of which occurred with the 0.18-mm. wire. For the 146 flights selected for analysis the bat's position was determined at the time each pulse was emitted, and the pattern of sound emission in typical flights is shown graphically in Figures 2–6, where each point represents a single pulse. Since the repetition rate varies rapidly it is more appropriate to consider the data in terms of the time interval between pulses. Therefore the or-

dinate of these graphs shows the time elapsed since the previous pulse, together with the corresponding repetition rate. Figures 2–6 show typical examples of these curves with five of the six sizes of wire studied, including cases when the pulse-to-pulse interval was both relatively constant (Fig. 6) or rather variable (Fig. 3) before the approach to the wires, cases in which the actual values of the interval were high (Fig. 2) or low (Fig. 3), and cases in which the drop was slight (Fig. 5) as well as others in which it was very marked (Fig. 4).

In the present experiments the wires were hung from small screw hooks in the ceiling, but the vocal reactions occurred when the bats were flying more than a meter below the ceiling, and thus were most unlikely to be reacting to the hooks. Further-

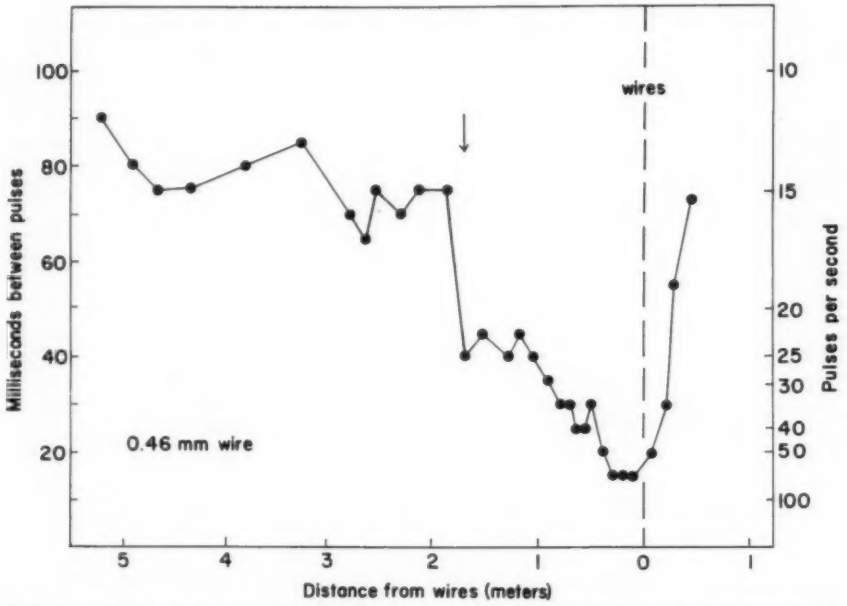

FIGURE 4. Intervals between pulses emitted by a bat flying through a row of 0.46-mm. wires.

more, there were many similar hooks elsewhere on the ceiling which caused no change in the emitted sounds, and control tests with no wires hanging from the hooks showed no significant variations in the pulse-to-pulse interval. Each of fourteen bats for which clear records of the first flights are available yielded a typical curve like those shown in Figures 2–6 the first time it flew through the experimental room, showing that the change in pulse repetition pattern was not merely the result of familiarity with the position of the wires.

The actual values of the pulse-to-pulse interval varied considerably. With a few individuals it was as high as 150–170 msec during the straight flight towards the wires (Fig. 2), with others it was approximately constant at 40 to 60 msec (Fig. 3). Just at the wires the interval sometimes fell to about 10 msec, but in

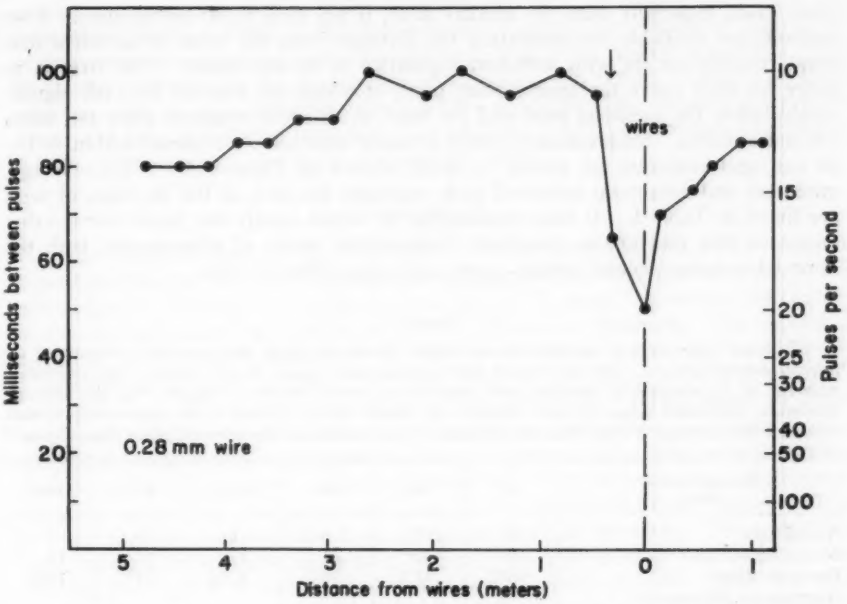

FIGURE 5. Intervals between pulses emitted by a bat flying through a row of 0.28-mm. wires.

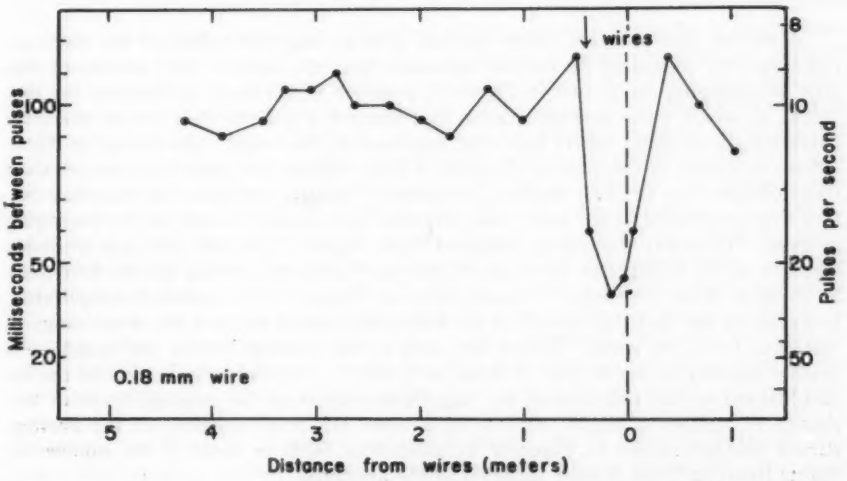

FIGURE 6. Intervals between pulses emitted by a bat flying through a row of 0.18-mm. wires.

other cases, especially with the smaller sizes, it fell only to 40 or 50 msec. Two methods are available for estimating the distance from the wires at which a first vocal reaction occurs with sufficient regularity to be significant. The first is to judge for each curve the approximate point at which the interval first fell significantly below the previous level and the level to which it returned after the wires had been passed. Such estimates could be made with some confidence within ± 15–20 cm., and examples are shown by small arrows on Figures 2–6. The average, minimum and maximum values of such estimates for each of the six sizes of wire are listed in Table I. It was encouraging to obtain nearly the same average distances of first reaction in completely independent series of photographs with the same wires taken several months apart and using different bats.

TABLE I

Distance from wires of various sizes at which Myotis lucifugus first reacted by decreasing the interval between pulses. The wires used were vertical and spaced 30 cm. apart. The individual estimates of the distance of detection were made from curves similar to Figures 2–6; and average, minimum, maximum values of such estimates are shown below. Owing to the uncertainty of such estimates their average is lower than the distance of first reaction to the wires obtained from Figure 7

Diameter of wire (mm.)	3.0	1.07	0.65	0.46	0.28	0.18
No. of bats	10	17	6	6	3	3
No. of flights	29	42	21	17	13	19
Per cent misses	93%	97%	76%	82%	77%	74%
Average est. distance of detection (cm.)	186	144	133	118	92	66
Range	(66–294)	(49–197)	(66–245)	(69–180)	(30–138)	(30–117)
Distance of detection obtained from Fig. 7 (cm.)	215	185	150	120	105	90

A second, and probably better, method is to average the values of the pulse-to-pulse interval measured at various distances from the wires. The results of this type of averaging are shown in Figure 7, together with arrows to indicate the distances at which these average curves first showed a definite drop below the level characteristic of flight before and after approach to the wires. As shown in Table I these estimates of the average distance of first reaction are somewhat greater than those obtained by the first method, presumably because variation due to other factors than proximity to the wires was cancelled out to some extent in the averaging process. Therefore the values obtained from Figure 7 provide the best available measure of the distance at which alert and successful bats of this species first react to wires of these six sizes. Consideration of Figure 7 is somewhat complicated, however, by the fact that not all of the individual curves covered the same range of distances from the wires. Hence the ends of the average curves are based on a smaller number of flights than is listed in Table I. Careful examination of the individual curves did not disclose any significant effects on the average curve of this change in number of flights, and in those more important portions of the average curves that are shown in Figure 7 by solid dots, 90% or more of the number of flights listed in Table I were included in the averages.

Table I shows that with all six sizes of wire there was a large proportion of

misses, the remaining flights being "touches" or "hits" as defined by Griffin and Galambos (1941). Study of the few individual curves for touches or hits in the present series showed no appreciable difference from those ending in a miss. This is not inconsistent with the observation of Galambos and Griffin (1942) that there was less likely to be a change in rate in flights ending in a hit, because the present series was initially selected to include only straight flights by bats that were registering a high degree of success at dodging the wires.

Discussion

These measurements are an extension of earlier experiments in which the pulse repetition rate was shown to increase as bats approached wires that were about 1.2 millimeters in diameter. It is therefore important to point out certain differences in the methods used and in some aspects of the results obtained. The apparatus available for the earlier studies was not capable of reliably registering bat pulses at a sufficient distance to provide information such as that presented in Figures 2–7, even if the bat's distance from the wires had been recorded. Furthermore the room was smaller (4.5 meters long instead of 10 meters in the present experiments), and the flights studied were not limited to straight approaches by bats at their optimal level of skill at echolocation. This is probably why a higher proportion of the present series were misses, and why almost every one of the present trials showed a clear decrease in pulse-to-pulse interval as the bat approached the wires. More sensitive apparatus might well have revealed a larger proportion of cases with a slight but detectable change in repetition rate, had it been available in 1941. But this does not alter the fact, demonstrated at that time, that successful bats are much more likely to show a marked change in repetition rate on approaching small obstacles than are those which collide with the wires.

In the original experiments it was our impression from visual observation that the bats ordinarily reverted to a distinctly lower pulse repetition rate just *before* passing through the wires. It is therefore of interest to examine the more extensive and accurate data obtained in the present experiments with respect to the positions at which the pulse-to-pulse interval rose again to approximately the value measured before the bat approached within two meters of the wires. It is clear from the individual flights illustrated in Figures 2–6, as well as from the average curve for each size of wire, that the interval did not completely return to its earlier level until some distance past the wires. In several individual curves, however, the pulse-to-pulse interval appears to have risen shortly before the wires were passed, as in Figure 3. But many other individual curves, such as Figures 2 and 4, show that the pulse-to-pulse interval did not rise appreciably until the wires had been passed. It should be recalled in this connection that the position of the bat was determined only within ± 10–15 cm., and in a majority of individual curves the increase in interval occurred within this distance of the wires. In a substantial minority of cases the first definite increase appeared to be delayed until the bat was more than 15 cm. past the wires (8 out of 29 flights with 3-mm. wire, 17 out of 42 with 1.07-mm., 8 out of 21 with 0.65-mm., 4 out of 13 with 0.46-mm., but only one out of 13 with the 0.28-mm. and 3 out of 19 flights with the 0.18-mm. wire). In only one case, with the 0.28-mm. wire, the curve began to rise more than 15 cm. before the plane of the wires.

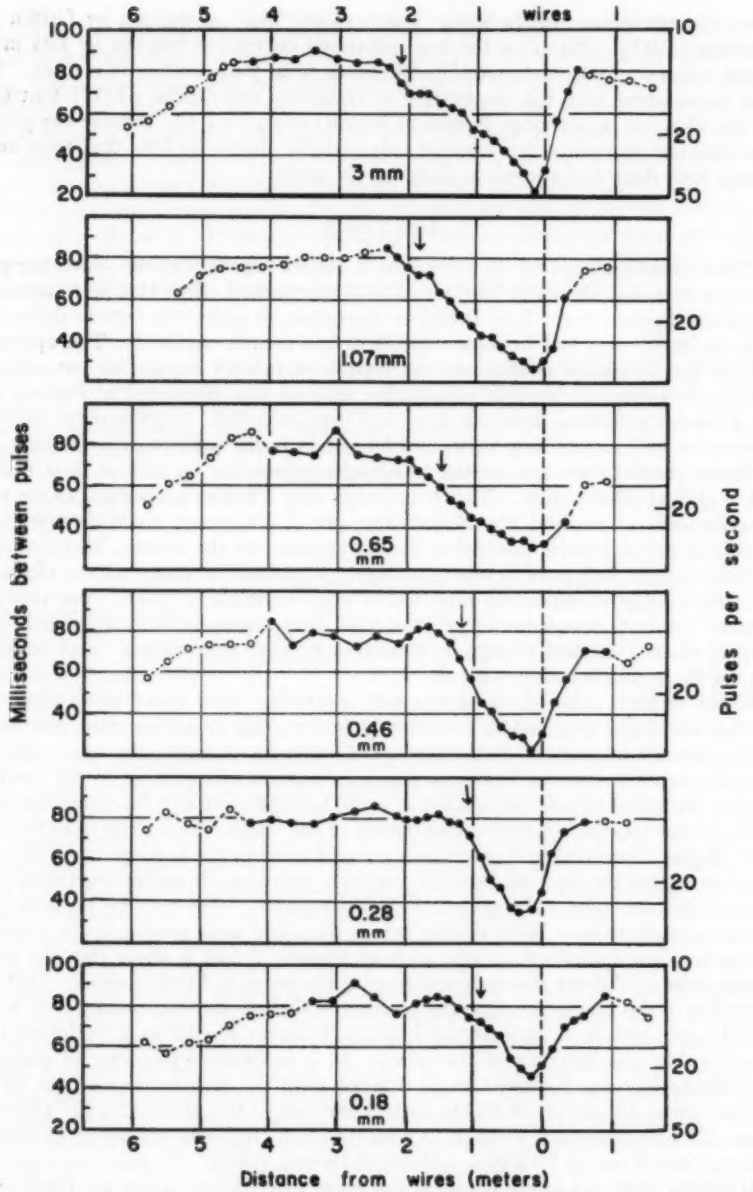

FIGURE 7. Average pulse-to-pulse intervals of bats flying past wires ranging from 0.18 to 3 mm. in diameter. The arrows indicate the distance of first vocal reaction.

The average curves of Figure 7 show a slight increase in the interval just as the bats flew past the wires. But since the possible error of the determinations of the bat's position was ± 10–15 cm. this small difference is only barely significant. In short, these measurements demonstrate only that the rise in the interval between pulses occurred on the average within 15 cm. of the wires, and was apparently more likely to begin shortly before passage through the plane of the wires than shortly after. Yet the 1.07-mm. wire was approximately the same size as the wires used in the earlier tests, and the spacing of the wires was the same. It is not clear whether the difference between our strong impression from observing the first experiments and the results of these more accurate measurements resulted from the selection of more alert and skillful bats for the present series, from the larger size of the room, or from some other factor.

In many flights the pulse-to-pulse interval just before the bat reached the wires alternated somewhat regularly between two quite different values, as in Figure 2. In other words the pulses tended to come in pairs, each pair separated from the

TABLE II

Number of flights showing a marked alternation in the interval between pulses, similar to that illustrated in Figure 2

Diameter of wire (mm.)	Definite alternation		No alternation		Doubtful	
	Number	Per cent	Number	Per cent	Number	Per cent
3.0	25	86%	2	7%	2	7%
1.07	23	55%	11	26%	8	19%
0.65	12	57%	4	19%	5	24%
0.46	5	26%	10	53%	2	11%
0.28	1	8%	10	77%	2	15%
0.18	2	10.5%	15	79%	2	10.5%

next by an interval somewhat greater than that between the members of each pair. A similar tendency for pulses to occur in groups of two, three or occasionally four, was apparent in the first graphic records of bats' orientation pulses (Galambos and Griffin, 1942). Perhaps these groups correspond to the respiratory cycle. In the present series of curves the presence of this feature is clearly correlated with the size of the wires themselves, as shown in Table II.

Whatever significance this alternation may have, it was more likely to occur with the larger sizes of wire. Perhaps there was simply not time during the last quarter of a second or so of flight up to the 0.18- or 0.28-mm. wire for so complicated a vocal reaction. Indeed, if the decrease in pulse-to-pulse interval did not occur until the bat was closer than one meter to the wires, the period of increased repetition rate often contained only five or six pulses. Whatever additional information the bat obtained from the extra pulses over and above those that would have been produced at the previous rate, its vocal reaction was a brief and limited one.

The pattern of sound emission has been discussed above in terms of time, but it is also of interest to consider it in terms of space. The same photographs show how fast the bats were moving towards and past the wires, and the average of 54

velocity measurements was 3.9 meters per second, with the extremes of the series 2.4 and 6.3 meters/second. The speed did not vary significantly with the different sizes of wire, nor at different portions of the individual flights, except in a few cases when a late turn to dodge a wire caused momentary slowing and fluttering. Since the interval between pulses averaged about 70 milliseconds before the repetition rate had increased in proximity to the wires, a flight velocity of four meters per second means that one pulse was emitted about every 28 cm. As the bats flew within 0.5 meter of the wires the interval often fell to 20–30 msec, and the lower figure corresponds to one pulse every 8 cm. of flight at 4 meters/sec., or approximately once every time the bat moved through a distance equal to its own length. When the flight slowed in front of the wires, even shorter distances separated the positions at which successive pulses were emitted.

The actual detection of an echo from the wires must of course have preceded the first vocal reaction of the bat, and hence the distance of detection was somewhat greater than the distance of reaction discussed above. To consider the 3-mm. wire, for example, the average distance of reaction was 215 cm. But the first pulse to occur after a shortened interval was not registered until it had travelled to the microphone located 210 cm. beyond the wires, or 425 cm. from the bat. The acoustic delay for this distance is about 13 msec. A further correction should be made for the bat's reaction time, which might have been as little as 15 msec (the approximate time required for the contraction of the intra-aural muscles at the onset of a loud sound), or perhaps it was as long as 200 msec (the order of magnitude of minimum human reaction times). A conservative estimate of 25 msec for the sum of acoustic delay and reaction time indicates that when a bat detected the echoes of the 3-mm. wires it was at least 10 cm. farther from the wires than our data demonstrate directly. A similar estimate for the 0.18-mm. wires places the bat about 100 cm. when their echoes first became audible. If this does not appear to be a very impressive range of detection it is well to bear in mind that it is about 5500 times the diameter of the wires themselves.

The success of bats in avoiding wires naturally varies with the diameter of the wire; but even when wires are spaced 30 cm. apart the percentage of misses of an alert *Myotis lucifugus* does not fall sharply until the diameter of the wire is reduced below about 0.3 mm. In a long series of experiments with this species performed by Curtis (1952) in a smaller flight room, the average percentage of misses of wires spaced the same distance apart varied as follows with the wire diameter: 4.8 mm., 85%, 1.21 mm., 85%, 0.68 mm., 77%, 0.35 mm., 72%, 0.26 mm., 52%, 0.12 mm., 39%, and 0.07 mm., 36%. The chance score at this spacing is about 35%. These bats had been less highly selected for skillful flight and obstacle avoidance than those in the present series. For example, the three bats tested with 0.18-mm. wire registered 32 misses out of 46 flights photographed. The only selection involved in this series was the decision that the bat was flying well and tending to head straight towards the wires so that it was worthwhile to take pictures of it.

In view of the small size of the wires, relative to the wave-lengths of the emitted sounds, it is surprising that the distance of detection did not vary more with wire size. While the ratio of wire diameters was about 17:1, the distances of reaction varied only by less than 2.5:1. If Raleigh scattering was the chief source of the echoes, the ratio of echo intensities at a fixed distance should have been $(17)^4$:1

(Morse, 1948). To be sure, the echo intensity also varies inversely as the cube of the distance (since the echo radiates from wires as a series of cylindrical waves), and atmospheric attenuation reduces the echo somewhat further. Even if we assume the echo intensity to vary inversely as the fourth power of the distance, we still face a puzzling discrepancy of $(17)^4/(2.5)^4$ or more than two thousand.

One way to escape from this dilemma is to postulate that much higher frequencies or shorter wave-lengths are used to detect these small wires, perhaps harmonics of the fundamental frequencies in the bat's orientation pulse. This might bring the wire diameters up to the order of one wave-length so that Raleigh scattering would not predominate, and the echo intensity would vary more slowly with wire diameter. But all available evidence indicates that the maximum intensity of the emitted pulse, and the maximum sensitivity of hearing, both occur at about 50–60 kc, or a wave-length of 6 to 7 mm., where all but the two larger sizes should be within the range of Raleigh scattering. At higher frequencies the echo intensity and the sensitivity of hearing would probably both fall off fairly rapidly.

Another and perhaps better explanation would be that the bats could actually detect the wires at greater distances than our data indicate, but that they do not trouble themselves to increase the pulse repetition rate until they come within a meter or two. The relatively small increase in distance of vocal reaction between the 1.07- and 3-mm. wire could be explained about equally well by assuming that the echo strength increased less rapidly as the wire diameter approached one wave-length, or by postulating that the 3-mm. wire was detected at a greater distance but did not elicit a vocal reaction until about two meters. We cannot resolve this question without new and more refined experimental evidence. It is interesting to note in this connection a suggestion of a double break in the curves for the 1.07- and 3-mm. wires. It is possible that a slight reduction in the pulse-to-pulse interval occurs somewhat earlier than the onset of the pronounced drop which is apparent at all six wire sizes. About one-third of the individual curves for the 3-mm. wire have a distinct double break, as shown, for example, in Figure 2.

Since these insectivorous bats apparently detect small wires at 1.0 to 2.25 meters it is natural to inquire whether larger objects can be located at correspondingly greater distances. One factor which limits a simple extrapolation to larger sizes and longer ranges of detection is the attenuation of high frequency sound in air. (For values of the coefficient of atmospheric absorption in the bats' frequency range see Beranek, 1949, pages 64–72.) Furthermore the intensity of the echo falls off as approximately the third or fourth power of the distance, depending upon the geometry of the object reflecting or scattering the sound. Nevertheless it must be possible for these bats to detect objects several centimeters in size at considerably greater distances, and really large objects such as trees or buildings are presumably detectable at distances of many meters. No methods have yet been devised, however, to determine objectively the maximum distances at which such objects are first detected by bats, and this fact presents a real challenge to future students of echolocation and bat behavior.

Summary

1. The distance at which bats (*Myotis lucifugus*) react to the presence of a row of small wires has been measured by a photographic determination of the

distance at which the pulse repetition rate first increases as the bats fly towards the wires. Distinct changes in this rate were measured in almost every flight towards wires spaced 30 cm. apart and ranging in diameter from 0.18 to 3 mm.

2. The interval between successive pulses averaged 60 to 80 msec as the bats flew along the room towards the row of wires, and dropped to 20–40 msec just before the barrier. The intervals decreased less with the smaller sizes of wire.

3. All but the largest of these wires are well below one wave-length of the emitted sounds of these bats (50–60 kc, or 6–7 mm., at the peak intensity and 120 kc, or about 3 mm., at the very beginning of some pulses).

4. Clear evidence that the wires had been detected was furnished at the point where the interval between pulses first dropped significantly below the level that prevailed before and after the approach to the row of wires. This average distance of first vocal reaction to the row of wires was 215 cm. for 3-mm. wire, 185 cm. for 1.07-mm., 150 cm. for 0.65-mm., 120 for 0.54-mm., 105 cm. for 0.28-mm., and 90 cm. for 0.18-mm. A conservative correction for reaction time and the acoustic delay between the bat and the microphone indicates that the distance of first detection must have been at least 10 cm. greater than these distances of reaction.

5. Since small wires can be detected at distances of as much as 5500 times the wire diameter, and well before the bat gives evidence by its flight pattern that it is aware of them, it appears likely that larger objects are detected at considerably greater distances.

LITERATURE CITED

BERANEK, L. L., 1949. Acoustic measurements. John Wiley and Sons, New York.

CURTIS, W. E., 1952. Quantitative studies of echolocation and vision in bats and owls. Thesis deposited in Library of Cornell University, Ithaca, N. Y.

GALAMBOS, R., AND D. R. GRIFFIN, 1942. Obstacle avoidance by flying bats; the cries of bats. *J. Exp. Zool.*, **89**: 475–490.

GRIFFIN, D. R., 1950. Measurements of the ultrasonic cries of bats. *J. Acoust. Soc. Amer.*, **22**: 247–255.

GRIFFIN, D. R., 1953. Bat sounds under natural conditions, with evidence for the echolocation of insect prey. *J. Exp. Zool.*, **123**: 435–466.

GRIFFIN, D. R., 1958. Listening in the dark. Yale University Press, New Haven, Conn.

GRIFFIN, D. R., AND R. GALAMBOS, 1941. The sensory basis of obstacle avoidance by flying bats. *J. Exp. Zool.*, **86**: 481–506.

GRIFFIN, D. R., AND A. NOVICK, 1955. Acoustic orientation of neotropical bats. *J. Exp. Zool.*, **130**: 251–300.

MÖHRES, F. P., 1953. Über die Ultraschallorientierung der Hufeisennasen (Chiroptera-Rhinolophidae). *Zeitschr. f. vergl. Physiol.*, **34**: 547–588.

MORSE, P. M., 1948. Vibration and sound. McGraw Hill Book Co., New York. Sec. Edit.

PHYSIOLOGY OF INSECT DIAPAUSE. XI. CYANIDE-SENSITIVITY OF THE HEARTBEAT OF THE CECROPIA SILKWORM, WITH SPECIAL REFERENCE TO THE ANAEROBIC CAPACITY OF THE HEART[1]

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Among the metabolic changes which accompany the onset of insect diapause is a pronounced decrease in sensitivity to cyanide and carbon monoxide. This fact was first discovered by Bodine and Boell (1934a, 1934b) in diapausing eggs of the grasshopper *Melanoplus*, and has subsequently been studied in further detail in *Melanoplus* (Robbie *et al.*, 1938; Robbie, 1941) and in the Cecropia silkworm. The situation in the case of Cecropia may be summarized as follows.

Cyanide and carbon monoxide are lethal agents for the caterpillar of the Cecropia silkworm—a fact which mirrors the presence in the larval insect of an intact and functional cytochrome system. However, immediately after pupation the cytochrome system undergoes partial breakdown in all tissues except the intersegmental muscles of the abdomen. Simultaneously, the over-all metabolism of the diapausing pupa becomes substantially insensitive to cyanide and high pressures of carbon monoxide. This state of affairs persists throughout the prolonged period of pupal diapause. Finally, months later, the termination of diapause and initiation of adult development are accompanied by re-synthesis of cytochromes and the appearance of a fresh increment of metabolism which is sensitive to carbon monoxide. If one blocks this increment with cyanide or carbon monoxide, the insect's development is brought to a standstill.

On the basis of these findings one may infer that the metabolism during the growing, non-diapausing stages in the life history is mediated by the usual cyanide- and carbon monoxide-sensitive cytochrome oxidase. In this sense there is nothing remarkable about the insect's metabolism before and after the pupal diapause. But what is remarkable is the character of the metabolism of the diapausing insect itself. The clear-cut resistance to cyanide and carbon monoxide suggests that the metabolism of the diapausing insect proceeds *via* some simpler and more primordial system of electron transfer making use of a terminal oxidase other than cytochrome oxidase. Under this point of view, the metabolism of the diapausing pupa is conceived to differ, not only quantitatively, but also qualitatively, from that before and after diapause. This prospect has been examined experimentally by Schneiderman and Williams (1954a, 1954b) and incorporated into a comprehensive theory of the biochemistry of diapause.

Crucial to this interpretation is the breakdown of the cytochrome system at

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the outset of pupal diapause—a matter which has recently been re-examined by Shappirio and Williams (1957a, 1957b) in individual tissues of the Cecropia silkworm. Spectrophotometric studies at room temperature and spectroscopic studies at the temperature of liquid nitrogen confirm that, in all tissues except the intersegmental muscles of the abdomen, rapid changes in the cytochrome system take place at the outset of pupal diapause. Within 24 hours after the pupal molt, cytochromes *b* and *c* decrease at least 30-fold and become indetectable; cytochrome b_5 and cytochrome oxidase ($a + a_3$) likewise decrease at this same time, but then stabilize at low but finite levels. The net effect is that throughout the pupal diapause the tissues contain cytochrome oxidase in large excess over cytochrome *c*. Consequently, if the cyanide- and carbon monoxide-sensitive system fails to participate in the metabolism of the diapausing tissues, then the block in electron transfer must be localized at the level of cytochrome *c* rather than at the level of cytochrome oxidase itself.

Whether cytochrome *c* actually disappears at the outset of diapause is a matter which lies beyond the resolution of the most sensititive methods of assay available at the present time. This is a question of decisive importance because a low concentration of *c* in the presence of a tremendous excess of oxidase might camouflage the participation of the cytochrome oxidase system in the metabolism of diapause. Thus, by means of carbon monoxide or cyanide one could poison, say, 95 per cent of cytochrome oxidase activity and the residual 5 per cent of active oxidase might still be able to saturate cytochrome *c* and sustain the low and "carbon monoxide-insensitive" metabolism of the diapausing insect.

Because of the limitation inherent in methods for the assay of cytochrome *c*, the problem appeared to be intractable to further biochemical analysis at the present time. Therefore, we have directed attention back to the insect itself. We have selected for intensive study the physiology of a particular tissue, the insect heart. Through an investigation of this tissue we have been able to bypass many of the above-mentioned difficulties and to comprehend what appears to be the mechanism of cyanide and carbon monoxide-sensitivity and -insensitivity in the Cecropia silkworm. In the present paper attention is directed to the effects of cyanide on the heartbeat of the insect during metamorphosis. In the following paper (Harvey and Williams, 1958) the effects of carbon monoxide will be considered.

Materials and Methods

1. *Experimental animals*

The experimental animals, *Platysamia cecropia* L., were reared and managed according to methods described previously (Williams, 1946, 1956). Experiments were performed on the insect at the following stages: mature larvae shortly before the initiation of spinning; unchilled pupae which had been stored at 25° C.; chilled pupae which had been stored at 6° C.; chilled pupae which had been stored for 4 to 6 months at 6° C. and then returned to 25° C. for one week; post-diapausing individuals at successive stages in adult development at 25° C.; and adult moths which had developed and emerged at 25° C. Certain experiments were performed in parallel on the related Polyphemus silkworm (*Telea polyphemus* Cram.).

2. Methods

A. *Exposure of isolated hearts to increasing concentrations of cyanide*

The dorsal half of the abdomen was excised with scissors and pinned by its lateral margins to a wax layer in the bottom of a circular dish of Lucite (polymethyl methacrylate). Each dish was provided with a Lucite cover and with inlet and outlet tubes arranged in such a manner that the preparation was automatically bathed in 20 ml. of gently flowing insect Ringer's solution (Ephrussi and Beadle, 1936). The latter was slightly modified by the substitution of 0.001 *M* potassium phosphate buffer (pH 7.0) for a corresponding proportion of the potassium chloride. To the solution prior to use were added a few milligrams of a 1:1 mixture of crystalline phenylthiourea and streptomycin sulfate—the former to block tyrosinase activity and the latter to oppose bacterial growth.

The gut and gonads were removed from the preparation, thereby exposing the heart and alary muscles. The paired masses of fat body were pressed aside so that the heart could be viewed *in situ* through a dissecting microscope.

The physiological solution was aerated continuously by a gentle stream of oxygen introduced into the fluid by a 20-gauge hypodermic needle passing through the lateral wall of the dish. A 26-gauge needle passing through the plastic cover permitted the addition of a solution of hydrogen cyanide; a reservoir of the latter was stored in a one-liter Pyrex wash bottle which was connected by Tygon tubing to the hypodermic needle.

The preparation was first equilibrated with insect Ringer until the heartbeat was stabilized. This ordinarily required one to two hours. The flow of Ringer was stopped and the cyanide solution was then dripped into the perfusion fluid at a rate of approximately ten drops per minute. The concentration of cyanide in this stock solution was 10 to 100 times the inhibitory level, as ascertained in preliminary experiments. The dropwise addition of cyanide was continued until the heartbeat was strongly inhibited. The oxygen flow was shut off and a two-ml. sample of the perfusion fluid was then immediately withdrawn into a hypodermic syringe and analyzed for cyanide by the phenolphthalin technique described by Robbie (1944).

B. *Exposure of isolated hearts in a flowing system*

An elongate plastic tube, 1.9 cm. in outside diameter, was cut longitudinally to form two semi-cylindrical troughs. The depression was then filled with melted wax. A series of hearts was isolated and pinned to the wax bottom of the plastic trough; the latter was then slipped into a glass tube (60 cm. long and I. D. 2 cm.). The glass tube was equipped with ground glass joints at its two ends. One end was connected by the ground joint to a stoppered reservoir containing the solution to be tested. The latter was forced from the reservoir by a slight positive pressure of overlying oxygen or nitrogen. The solution, after flowing slowly over the abdomens, made exit from the ground joint at the distal end of the glass tube and was passed in rubber tubing into a five-gallon bottle containing strong alkali. As the occasion required, samples of solution were withdrawn from the rubber tube with a hypodermic syringe and analyzed for cyanide or for oxygen.

C. *Appraisal of heartbeat*

In Method A the constant agitation of the oxygen bubbles caused considerable irregularity in the frequency of beat. Therefore, in appraising the heartbeat in experiments utilizing Method A, primary attention was centered on the amplitude of the beat rather than its frequency. This method of study was soon abandoned in favor of Method B. Here the frequency of heartbeat was found to be far more constant and predictable. Under constant conditions the variability of heart rate was small compared to that brought about by the experimental treatment. Routinely the frequency of heartbeat was counted for each individual over a period of from one to five minutes and averaged as beats per minute. The strength (amplitude) of the beat was also scored as normal (3), subnormal (2), barely detectable (1), and absent (0). In order to obtain an over-all index of heart function, the recorded frequencies were divided by 1 when the heartbeat was normal, by 2 when the beat was subnormal, and by 3 when the beat was barely detectable. We shall hereafter refer to this value as the "heartbeat index."

D. *Reagents*

Cyanide was obtained as potassium cyanide (Mallinckrodt) assaying not less than 96.0%. Fresh solutions were prepared daily in oxygenated Ringer, neutralized with 1 N hydrochloric acid to pH 7.0, and stored in stoppered containers in the cold. At this pH, 98% of the cyanide is present as hydrocyanic acid.

The experimental gases were obtained in pressure cylinders and assayed as follows: "pre-purified nitrogen" (Airco), 99.998%; oxygen (Airco), 99.5%.

Results

1. *Acute poisoning of the isolated heart*

Isolated hearts of Cecropia, at successive stages in metamorphosis, were exposed during a period of one-half hour to increasing concentrations of cyanide by Method A. The concentration required to inhibit the heartbeat during this half hour was ascertained for each of a series of animals at each of seven stages in metamorphosis. When judged in this manner, the cyanide-sensitivity of the heartbeat is found to undergo large and systematic changes during the course of metamorphosis.

As recorded in Table I and Figure 1, the heartbeat of the mature larva is blocked within 0.5 hour by cyanide concentrations somewhat less than 10^{-3} *M*. However, immediately after the pupal molt, a remarkable resistance to cyanide becomes evident. Thus, within one day after the molt, the inhibitory cyanide concentration increases to 5×10^{-3} *M*. This trend continues until, some two to three weeks later, the inhibitory concentration is not far short of 10^{-1} *M*. The net effect is that the transition of the larva into a diapausing pupa is accompanied by a 100-fold decrease in sensitivity to acute poisoning by cyanide. This condition then persists during the months of pupal diapause.

After prolonged exposure to 6° C., the pupal diapause is terminated; only one or two days at 25° C. are then required for the visible initiation of adult development (Williams, 1956). Though pupae of this type show no detectable development when examined immediately after their return to 25° C., it is of interest that

resistance to cyanide has already begun to decline (Table I and Fig. 1). By the first or second day of adult development the heart is approximately as sensitive to cyanide as the larval heart. During the three-week period of adult development at 25° C., one records an ever-increasing sensitivity to acute poisoning by cyanide. Finally, the heart of the freshly emerged adult moth is blocked by cyanide at concentrations as low as 10^{-5} *M*—a sensitivity 8,000 times that recorded for the diapausing pupa.

TABLE I

Acute toxicity of cyanide for Cecropia and Polyphemus hearts: cyanide concentrations which block the heartbeat during 0.5-hour exposure*

P. cecropia

Stage	No. of preparations	Final concentration of cyanide ($\times 10^{-4}$ *M*)	Reversibility of effects
Fifth instar larva	7	7.5 ± 0.46**	+
One-day-old pupa	6	50.0 ± 4.80	0
Pupa after 2–3 weeks at 25° C.	6	770.0 ± 170.00	0
Pupa after 8 months at 6° C.	12	77.0 ± 6.90	0
First or second day of adult development at 25° C.	5	5.1 ± 1.20	+
Fifteenth or sixteenth day of adult development at 25° C.	6	3.0 ± 1.70	+
Adult moth	21	0.1 ± 0.04	+

T. polyphemus

Stage	No. of preparations	Final concentration of cyanide ($\times 10^{-4}$ *M*)	Reversibility of effects
Pupa after 5 months at 25° C.	6	350.0 ± 44.00	0
Pupa after 7 months at 6° C.	11	31.0 ± 8.30	0
Eleventh or twelfth day of adult development at 25° C.	4	12.0 ± 3.10	0
Adult moth	8	0.5 ± 0.21	+

* No beat or only trace of beat during one minute of observation.
** Standard error.

As summarized in Table I, the observations were repeated on pupae and adults of the Polyphemus silkworm (*Telea polyphemus*). Here again, the cyanide resistance of the pupal heart is evident.

For both these species the response of the pupal hearts to acute poisoning by cyanide is remarkable, not only in terms of the high concentrations required to inhibit the heartbeat, but also in terms of the irreversibility of this inhibition (Table I). Whereas the heartbeat of larvae, developing adults, and adults is promptly re-

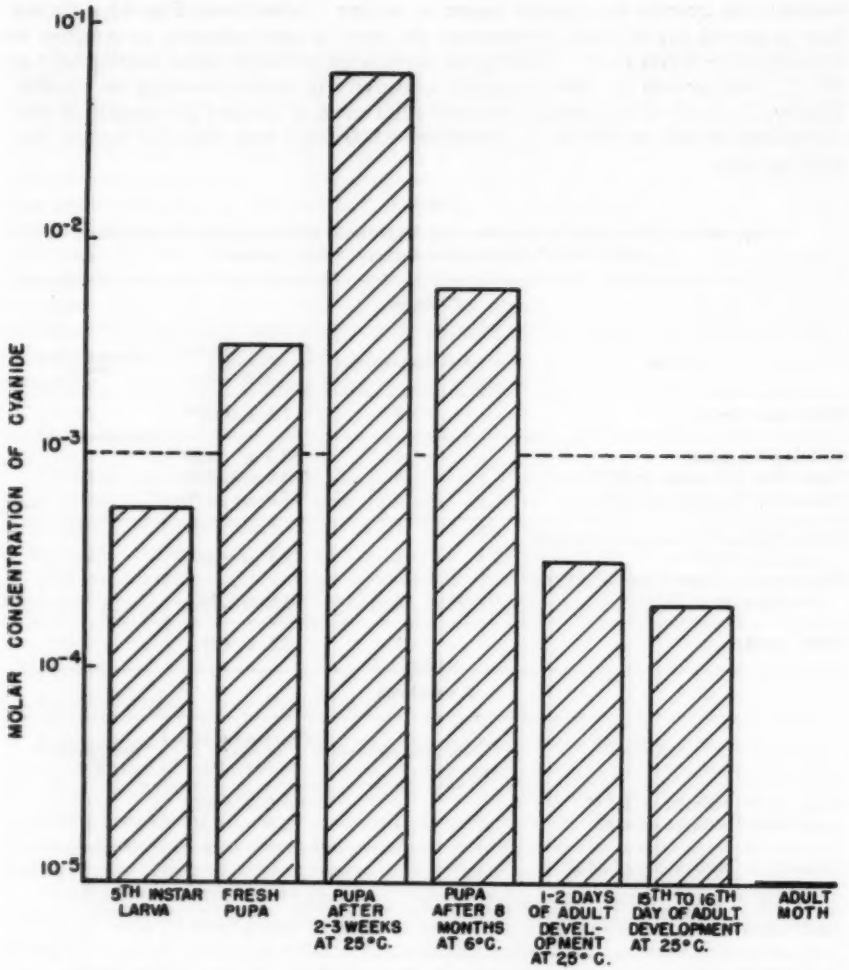

FIGURE 1. Cyanide concentrations required to block the beat of the isolated heart of the Cecropia silkworm within 0.5 hour. The resistance of the heart to acute cyanide-poisoning is seen to undergo major changes during the larval-pupal-adult transformation.

gained when returned to cyanide-free Ringer, the pupal hearts are evidently killed by the high concentrations required to inhibit them.

The experiment therefore directs attention to the paradoxical behavior of the pupal heart in relation to poisoning by cyanide. As illustrated in Figure 1, the pupal heart continues to beat normally for at least a half hour in cyanide concentrations far exceeding 10^{-3} M; that is, under conditions where one would anticipate

the inhibition of the vast majority of cytochrome oxidase activity. How can one account for this resistance of the pupal heart to cyanide?

One possibility is that the pupal heart contains a terminal oxidase other than cytochrome oxidase, and that this unknown oxidase is insensitive to cyanide. However, it was necessary to consider an even simpler explanation; namely, that the pupal heartbeat can be sustained by strictly anaerobic processes.

2. Pupal heartbeat under anaerobic conditions

The hearts of four diapausing pupae were isolated and pinned in the bottom of the glass tube described under Method B above. The tube was first perfused with a gently flowing stream of oxygenated Ringer, and the heartbeat of each individual ascertained. The 400-ml. tube was then perfused rapidly with oxygen-free insect Ringer; the perfusion was then continued at the lower rate of approximately 500 ml. per hour. Special attention was given to the total removal of oxygen from the physiological solution prior to its use. For this purpose, pre-purified nitrogen was bubbled through the Ringer for at least two hours; moreover, after traversing the solution the nitrogen was bubbled through a solution of reduced methylene blue (Fildes, 1931). The absence of color change gave assurance that all oxygen had been removed from the Ringer. The latter was then stored under a slight positive pressure of pre-purified nitrogen, and displaced by this pressure through the tube containing the hearts.

The hearts of four diapausing pupae were studied—first in air, then for 5½ hours in oxygen-free Ringer, and, finally, for 43 hours in oxygenated Ringer. The various measurements are summarized in Table II, along with the average heartbeat indices.

One is immediately impressed by the striking resistance of these diapausing hearts to strictly anaerobic conditions. After 0.5 hour of anaerobiosis, none of the hearts showed any detectable depression. After one hour, only one of the four was depressed. Between the first and second hours the over-all index value decreased

TABLE II

Effects of strictly anaerobic conditions on isolated hearts of brainless diapausing Cecropia pupae

Animal No.	Rate (beats/min.) and amplitude* of heartbeat										
	Air	Hours in Ringer equilibrated with pre-purified nitrogen						Subsequent hours in oxygenated Ringer			
		½	1	2	3	4½	5½	¼	1½	25	43
1	12 (3)	17 (3)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	8 (3)	22 (3)	17 (3)	10 (3)
2	13 (3)	13 (3)	12 (3)	12 (2)	13 (3)	5 (3)	4 (2)	14 (3)	17 (3)	13 (3)	9 (3)
3	17 (3)	19 (3)	19 (3)	13 (2)	0 (0)	14 (2)	0 (0)	20 (3)	4 (3)	6 (3)	7 (3)
4	12 (3)	20 (3)	19 (3)	14 (3)	12 (2)	8 (1)	0 (0)	26 (3)	19 (3)	13 (3)	8 (3)
Average heartbeat index	13.5	17.2	12.5	6.8	4.8	3.8	0.5	17.0	15.5	12.2	8.5

* See Methods.

markedly; however, one of the hearts showed a normal beat after two hours of anaerobiosis and another, after three hours. Three of the four hearts were still beating after 4½ hours of anaerobiosis, and one, after 5½ hours.

It will be observed in Table II that the effects of 5½ hours of anaerobiosis were promptly reversed when the hearts were returned to oxygenated Ringer. Indeed, the average index values actually increased for about an hour over the level at the

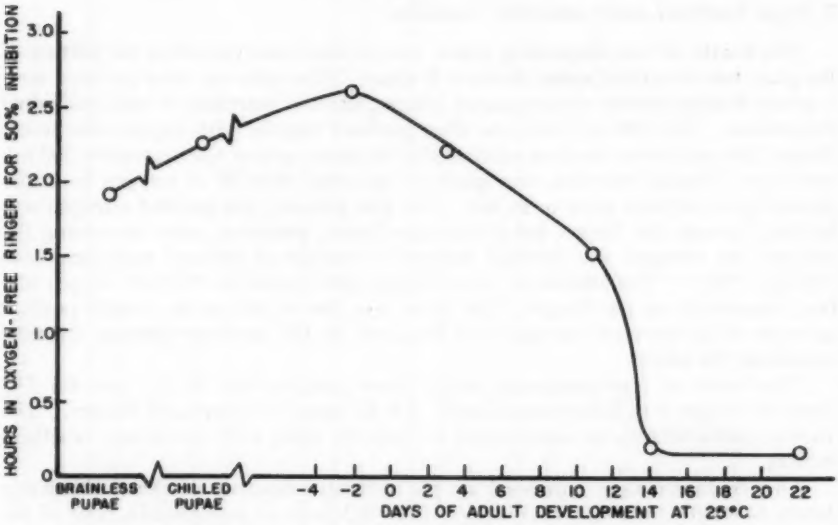

FIGURE 2. The "anaerobic reserve" of the isolated Cecropia heart is plotted as a function of pupal-adult development. The discontinuities in the curve correspond to days or weeks of storage under the conditions noted on the X-axis. The anaerobic reserve declines almost to zero during the course of adult development. Each datum is the average derived from the hearts of four to eleven individuals.

outset, and then stabilized at or near the initial, normal level. The absence of any permanent damage attributable to anaerobiosis is also confirmed by the continuation of heartbeat for 1½ further days until the experiment was abandoned.

3. *Heartbeat of chilled pupae, developing adults, and adults under anaerobic conditions*

Is the high degree of facultative anaerobism peculiar to the heart of the diapausing pupa? In order to answer this question the experiment, just considered, was repeated on the hearts of: previously chilled pupae; chilled pupae that had been returned to 25° C. and were just prior to the initiation of adult development; developing adults; and adults. The results are recorded in Figure 2 in terms of the period of anaerobiosis required for the reversible inhibition of 50 per cent of the average heartbeat index. The method of arriving at this value is illustrated in Figure 3. The average heartbeat indices of the diapausing pupae are here plotted

as a function of the duration of exposure to oxygen-free Ringer. A smooth curve is drawn by inspection through the series of points and the time for 50 per cent inhibition is ascertained from the curve.

In the results summarized in Figure 2, it is clear that a considerable capacity for anaerobism persists within the pupa during the months of chilling at 6° C. When pupae of this type are placed at room temperature, the capacity for anaerobism actually appears to increase slightly. By the fourth day of adult development the "anaerobic capacity" has returned to the level observed in diapausing pupae. This trend continues and by the eleventh day of adult development the time for 50 per cent inhibition under anaerobic conditions has dropped to 1.5 hours. On or about the eleventh day of adult development the anaerobic capacity decreases precipitously to the low level characteristic of the adult moth. The adult moth, emerging after 21 days of development at 25° C., is maximally sensitive to oxygen lack in that the heart is able to beat less than 10 minutes in the total absence of oxygen.

In the experiments just considered, the anaerobic condition was established by the use of oxygen-free Ringer's solution. The observations on pupal and adult hearts were repeated in a series of experiments in which the anaerobic condition was established by the ventilation of the tube with a flowing stream of pre-purified nitrogen gas (300 ml. per hour). Precisely the same results were observed.

In a further series of experiments making use of pre-purified nitrogen, the findings were confirmed in studies of the pupal and adult hearts of *Telea polyphemus*.

Consequently, for both these species, it is clear that the pupal heart, unlike the adult heart, possesses a substantial "anaerobic reserve" which can sustain the beating of the heart for as long as 5½ hours in the total absence of oxygen. Aside from the intrinsic interest of this new finding, the anaerobic capacity of the pupal heart is obviously critical in the design of experiments testing the aerobic metabolism of the pupal heart.

4. *Sensitivity of the pupal heart to prolonged exposure to cyanide*

In Section 1 the pupal heart was found to be extremely resistant to cyanide. However, it will be recalled that this result was based on experiments of short duration in which the heart was exposed to increasing concentrations of cyanide during a period of 0.5 hour. We now see that the pupal heart can beat for up to 5½ hours in the total absence of oxygen. The earlier experiments were therefore inadequate as a test of the cyanide sensitivity of the pupal heart. For this reason the effects of cyanide on the pupal heartbeat were re-examined in experiments of prolonged duration.

Isolated pupal hearts were placed in the flow tube (Method B) and subjected to a flowing stream of oxygenated insect Ringer containing a precise concentration of cyanide. The reservoir of Ringer was prepared in a stoppered five-gallon bottle and stored under oxygen. In order to cause the Ringer to flow through the experimental tube, the reservoir was slightly compressed by the addition of a stream of oxygen; the latter was bubbled through an aqueous solution of 10^{-1} or 10^{-2} *M* potassium cyanide before entering the reservoir. In this manner it was possible to prevent any significant change in the cyanide concentration in the Ringer during prolonged experiments. This fact was confirmed by cyanide assays performed on fluid that had traversed the chamber.

Cyanide at two specific final concentrations was studied in detail; namely, 10^{-2} *M* and 10^{-3} *M*. It will be recalled that both these concentrations were without detectable effects on the pupal heartbeat in experiments of short duration.

The effects of 10^{-2} *M* cyanide are summarized in Figure 4. In terms of the average index values, the heartbeat remained normal for 1½ hours. Two of the six hearts stopped beating after 2 hours. After 3½ hours, all hearts showed considerable depression, and three of the six had stopped. The average time required for 50 per cent inhibition was 2.25 hours. The tube was then flushed with cyanide-free Ringer. Three hearts showed a slight recovery at this time. This was a temporary effect, however, for all six hearts were in standstill after a total of two hours in cyanide-free Ringer.

In like manner the effects of perfusion with oxygenated Ringer containing 10^{-3} *M* cyanide were studied. A considerable depression was first evident after 2¾ hours, but all four hearts were still beating after 4 hours. At the end of 5¼ hours, two of the hearts stopped beating and the other two showed only a trace of beat. At this time the system was flushed with cyanide-free Ringer. All four hearts showed a delayed recovery and three of the four were beating normally after a total of 16 hours in cyanide-free Ringer.

Discussion

The heartbeat of the larva and the adult Cecropia silkworm is blocked in a reversible manner by brief exposure to cyanide in concentrations less than thousandth molar. Therefore, on the basis of this classical test, it seems safe to conclude that the hearts of the larva and the adult moth make use of cytochrome oxidase as "terminal oxidase."

When the same criterion is applied to the pupal heart, the latter is found to beat normally when immersed in cyanide at concentrations not far short of tenth molar. Here, then, is a tissue which appears to be totally insensitive to cyanide over the range of concentrations at which cyanide is an inhibitor of cytochrome oxidase. Consequently, the pupal heart has appeared to be a clear instance of a cyanide-insensitive tissue whose function is not dependent on metabolism mediated by cytochrome oxidase. Prior to the present investigation, we have routinely thought that this was so (Williams, 1951; Harvey and Williams, 1953).

On the basis of the experimental results described above, it is now clear that the pupal heart is by no means insensitive to cyanide. The crux of the matter is that the true cyanide-sensitivity of the heart is camouflaged most effectively by an anaerobic capacity peculiar to the pupal heart. Whereas the adult heart is able to beat for less than ten minutes in the total absence of oxygen, the pupal heart, by contrast, beats normally for one or more hours under the same conditions.

The experimental conditions leave little room for doubt that the pupal heartbeat, during this prolonged period of facultative anaerobism, is sustained by strictly anaerobic metabolism. Under this circumstance the heart can scarcely require the function of cytochrome oxidase or, for that matter, any other enzyme concerned with the utilization of atmospheric oxygen. Therefore, for a corresponding period the pupal heart is found to be totally insensitive to physiological concentrations of cyanide.

The true sensitivity of the pupal heart to cyanide is unmasked only when one

continues the experiment sufficiently long to use up the anaerobic reserve. This fact is evident in a comparison of Figures 3 and 4. The inhibition of the pupal heart by cyanide (Fig. 4) shows precisely the same time-course as that observed under anaerobic conditions in the absence of cyanide (Fig. 3). As the heart exhausts its anaerobic reserve, it becomes progressively more dependent on aerobic metabolism and progressively more sensitive to cyanide. These observations strongly argue that the aerobic metabolism of the diapausing heart requires the presence and function of cytochrome oxidase.

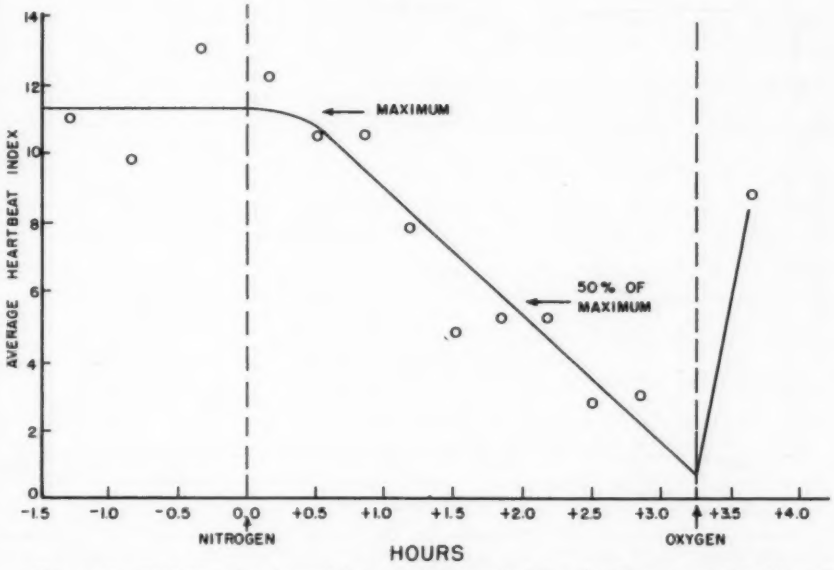

FIGURE 3. Technique for determining the time required for the 50 per cent inhibition of the heartbeat index during exposure of isolated hearts to oxygen-free Ringer. Each datum is the average from the hearts of eight brainless diapausing pupae.

On the basis of our present data we are unable to state the lower limit of cyanide concentration which inhibits the pupal heart in experiments of this type. However, for reasons which will be considered in detail in the following paper, we doubt that the pupal heart can ever be inhibited by the very low cyanide concentrations (10^{-5} M) which suffice to block the heartbeat of the adult moth.

While clarifying the problem of the cyanide-insensitivity of the pupal heart, the present study directs attention to a fresh problem—the changes occurring in the insect's capacity for anaerobic metabolism during the course of metamorphosis. As illustrated in Figure 2, these changes are large and systematic. Of particular interest is the rapid loss of "anaerobic reserve" which supervenes approximately midway in adult development.

We suspect that this change is not peculiar to the heart. Thus, according to Schneiderman and Williams (1954b), mature larvae and adult moths of the Ce-

cropia silkworm are killed in less than one day when exposed to "tank nitrogen" containing less than 0.5% oxygen. Diapausing pupae, by contrast, survive more prolonged exposures, the L.D. 50 per cent being three days.

On the basis of present inadequate information we are unable to comprehend the full meaning of this shift in the capacity for anaerobic metabolism. The quantita-

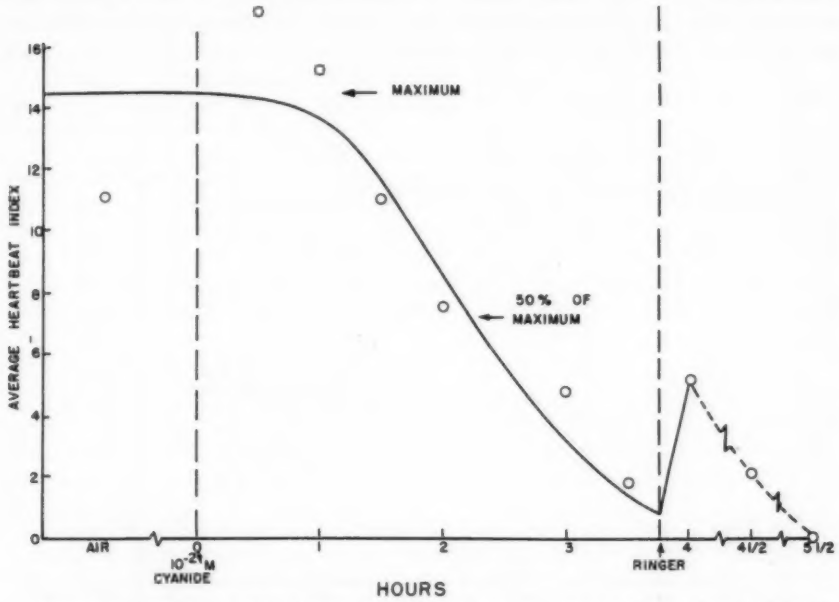

FIGURE 4. Effects of 0.01 *M* cyanide on the beat of hearts isolated from diapausing Cecropia pupae. Each datum is the average derived from the hearts of six brainless diapausing pupae.

tive changes suggested in Figure 2 are almost precisely the reverse of those occurring in the over-all aerobic metabolism and in the concentration of such typically aerobic enzymes as the cytochromes. The problem obviously merits further study.

SUMMARY

1. During the course of metamorphosis the heart of the Cecropia silkworm appears to undergo pronounced shifts in its sensitivity to cyanide.

2. In the mature larva the heartbeat is promptly blocked by 10^{-3} *M* cyanide; in the adult moth it is even more sensitive and is brought to a standstill by 10^{-5} *M* cyanide.

3. In the intervening pupal stage the heart is insensitive to acute poisoning by physiological concentrations of cyanide.

4. This insensitivity is observed only in experiments of short duration. When the exposure to cyanide is continued for many hours, the pupal heartbeat is blocked by 10^{-2} or 10^{-3} *M* cyanide.

5. The paradoxical response of the pupal heart can be accounted for in terms of a pronounced capacity for anaerobic metabolism which is peculiar to this particular stage. The pupal heart can beat for as long as 5½ hours in the complete absence of oxygen. During this same period the heart is insensitive to cyanide.

6. While discounting any true insensitivity of the pupal heart to cyanide, the experimental results direct attention to major and previously unsuspected changes in the anaerobic capacity of the Cecropia silkworm during the course of metamorphosis.

LITERATURE CITED

BODINE, J. H., AND E. J. BOELL, 1934a. Carbon monoxide and respiration: action of carbon monoxide on the respiration of normal and blocked embryonic cells (Orthoptera). *J. Cell. Comp. Physiol.*, **4**: 475–482.

BODINE, J. H., AND E. J. BOELL, 1934b. Respiratory mechanisms of normally developing and blocked embryonic cells (Orthoptera). *J. Cell. Comp. Physiol.*, **5**: 97–113.

EPHRUSSI, B., AND G. W. BEADLE, 1936. A technique of transplantation for *Drosophila*. *Amer. Nat.*, **52**: 218–225.

FILDES, P., 1931. Anaerobic cultivation. Chapter VI in System of Bacteriology, **9** (Gt. Brit.) Medical Research Council: 92–99.

HARVEY, W. R., AND C. M. WILLIAMS, 1953. Changes in the cyanide sensitivity of the heartbeat of the Cecropia silkworm during the course of metamorphosis. *Anat. Rec.*, **117**: 544.

HARVEY, W. R., AND C. M. WILLIAMS, 1958. Physiology of insect diapause. XII. The mechanism of carbon monoxide-sensitivity and -insensitivity during the pupal diapause of the Cecropia silkworm. *Biol. Bull.*, **114**: 36–53.

ROBBIE, W. A., 1941. The action of cyanide on eggs and embryos of the grasshopper, *Melanoplus differentialis*. *J. Cell. Comp. Physiol.*, **17**: 369–384.

ROBBIE, W. A., 1944. An improved phenolphthalin technique for the microdetermination of cyanide. *Arch. Biochem.*, **5**: 49–58.

ROBBIE, W. A., E. J. BOELL AND J. H. BODINE, 1938. A study of the mechanism of cyanide inhibition: I. Effect of concentration on the egg of *Melanoplus differentialis*. *Physiol. Zoöl.*, **11**: 54–62.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1954a. The physiology of insect diapause. VIII. Qualitative changes in the metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.*, **106**: 210–229.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1954b. The physiology of insect diapause. IX. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bull.*, **106**: 238–252.

SHAPPIRIO, D. G., AND C. M. WILLIAMS, 1957a. The cytochrome system of the Cecropia silkworm. I. Spectroscopic studies of individual tissues. *Proc. Roy. Soc. London, Ser. B*, **147**: 218–232.

SHAPPIRIO, D. G., AND C. M. WILLIAMS, 1957b. The cytochrome system of the Cecropia silkworm. II. Spectrophotometric studies of oxidative enzyme systems in the wing epithelium. *Proc. Roy. Soc. London, Ser. B*, **147**: 233–246.

WILLIAMS, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.*, **90**: 234–243.

WILLIAMS, C. M., 1951. Biochemical mechanisms in insect growth and metamorphosis. *Fed. Proc.*, **10**: 546–552.

WILLIAMS, C. M., 1956. Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the Cecropia silkworm. *Biol. Bull.*, **110**: 201–218.

PHYSIOLOGY OF INSECT DIAPAUSE. XII. THE MECHANISM OF CARBON MONOXIDE-SENSITIVITY AND -INSENSITIVITY DURING THE PUPAL DIAPAUSE OF THE CECROPIA SILKWORM [1]

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In the preceding paper of this series (Harvey and Williams, 1958), the effects of cyanide on the heartbeat of the Cecropia silkworm were studied during successive stages in metamorphosis. When suitable allowance was made for the anaerobic capacity of the diapausing pupa, the heartbeat at all stages was found to be sensitive to cyanide.

This finding, in itself, suggests that the heartbeat throughout the life history depends on the function of cytochrome oxidase. However, it will be recalled that cyanide combines with a number of enzymes in addition to cytochrome oxidase (Warburg, 1949). For this reason we have re-examined the matter making use of a far more specific inhibitor, carbon monoxide. In animals lacking hemoglobin, a light-reversible inhibition by carbon monoxide is sufficient proof of the presence and functional activity of cytochrome oxidase (Hill and Hartree, 1953).

Materials and Methods

1. *Experimental animals*

Diapausing pupae and adult moths of the giant silkworm, *Platysamia cecropia* L., were used as experimental animals. The diapausing individuals were of two types: pupae stored at 25° C. and utilized within three months after the pupal molt; and pupae stabilized in a permanent diapause by removal of the brain at least one month prior to use (Williams, 1946). In a number of cases, parallel experiments were performed on the related giant silkworm, *Telea polyphemus* Cram.

2. *Observations of hearts*

Due to surface reflection from the pupal cuticle, the heartbeat is not visible in the intact insect. Hearts were therefore studied after isolation as previously described (Harvey and Williams, 1958). Subsequently, in collaboration with Dr. Ned Feder, we found that the heart becomes plainly visible and can be studied in the intact pupa under the following circumstance. The light source is equipped with a polarizing filter. A second polarizing filter is placed below the objective of the dissecting microscope. When the planes of polarization are "crossed," surface

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reflection is totally eliminated. Except in very darkly pigmented individuals one can then clearly observe the beating heart.

3. Exposure of hearts to gases at elevated pressures

A series of from four to six hearts was isolated and pinned to an elongate paraffin-coated tray of Lucite. Ringer's solution (Ephrussi and Beadle, 1936), containing phenylthiourea and streptomycin sulfate, was then added until each heart was covered by a film of the solution. In experiments with intact pupae the insects were placed dorsal side up on a Lucite tray equipped with plastic cradles to accommodate the individual pupae.

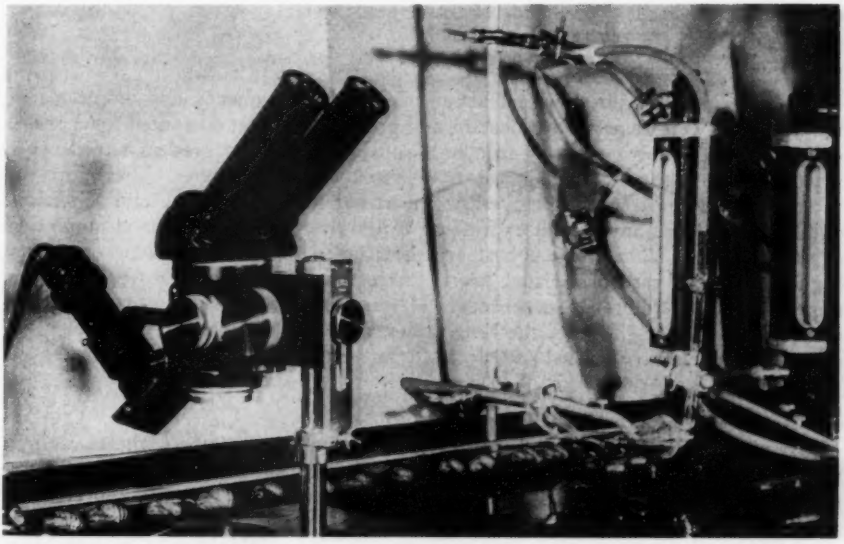


FIGURE 1. Apparatus for the exposure of pupae or of isolated hearts to flowing gas mixtures.

The tray with either isolated hearts or intact pupae was then inserted into a 3.5-liter Lucite chamber constructed to withstand high pressure (Schneiderman and Feder, 1954). The air-filled chamber was then sealed and compressed with carbon monoxide, the positive pressure being read from a gauge calibrated in pounds per square inch. The oxygen tension was therefore that of the initial air-filled chamber (21% atm.). In experiments utilizing oxygen pressures less than 21% atm., the chamber was first flushed with ten volumes of carbon monoxide. The system was then compressed with oxygen to a pre-determined pressure, making use of a mercury manometer. Finally, the chamber was further compressed with carbon monoxide.

4. Exposure of hearts to flowing mixtures of gases

As shown in Figure 1, intact pupae were placed in linear sequence in the depression of an elongate, semi-cylindrical tray of cellulose acetate and slipped into the

glass tube described under Method B by Harvey and Williams (1958). In experiments utilizing isolated hearts, the latter were pinned to a wax-coated tray and placed in the tube. The proximal end of the flow-tube was connected by a ground joint to a source of a constant flowing gas mixture; the distal end was connected by a ground joint to a length of rubber tubing which passed to a nearby chemical hood.

Specific mixtures of oxygen, nitrogen, carbon monoxide, and air were metered from Rotameters (Tri-Flat Variable-Area Flow Meters, Fisher and Porter, Co.). By selection of meters of appropriate capacity it was possible to utilize the several gases at rates of flow variable from 2 to 1000 cc. per minute, the error of each measurement not exceeding ten per cent. The several gas streams were combined and passed *via* rubber tubing to the experimental chamber, the total gas pressure being one atmosphere in all experiments of this type.

5. *Appraisal of heartbeat*

In experiments utilizing intact pupae, the average frequency and amplitude of the heartbeat were remarkably constant and predictable when computed for a series of individuals. The same was true to a somewhat lesser degree in experiments performed on isolated hearts in the flow-system described above. Therefore, in these types of preparations the average "heartbeat index" (Harvey and Williams, 1958) was used as an over-all expression of the average frequency and amplitude of the heartbeat. The frequency of heartbeat failed to show this same degree of regularity in the case of isolated hearts subjected to pressures greater than one atmosphere. Therefore, in experiments of this latter type, primary attention was centered on the amplitude of the beat rather than on frequency. Amplitude was scored on an arbitrary graded scale from 0 (no beat) to + + + (normal beat).

6. *Illumination*

By virtue of the transparent walls of the experimental chambers, the beating of the isolated heart was visible under the low magnification of the dissecting microscope. Incident illumination was utilized; namely, a focussed 15-watt lamp (Osram H3 6 volts) at a distance of approximately thirteen cm. from the preparation. In experiments pertaining to the light-reversibility of the inhibition, a second lamp (General Electric 1493, 30 watts) was also focussed on the same preparation. The lamps were equipped with one or more filters. An infrared filter (Corning No. 3962), was used routinely. Polarizing filters were used in studies of the intact animal (see above). And in experiments utilizing carbon monoxide mixtures, observations were performed in red light by the use of a Wratten filter (F filter No. 29).

7. *Reagents*

The experimental gases were obtained in compressed cylinders assaying as follows: "pre-purified nitrogen" (Airco), 99.998%; oxygen (Airco), 99.5%; anhydrous compressed air (New England Gas Products). The carbon monoxide was the Matheson product having the following composition: 96.8% carbon monoxide; 0.36% carbon dioxide; 0.97% hydrogen; 1% nitrogen; 0.8% saturated hydrocarbons; 1.19 mg. sulfur per liter. In order to minimize these several impurities, the gas was subdivided by sintered glass filters and bubbled through a succession

of two wash bottles containing 10 per cent potassium hydroxide; it then traversed a tube of anhydrous calcium chloride and passed to the flow-meters.

Results

1. *Effects of carbon monoxide on the heartbeat of pupae and adults*

The hearts of seven pupae and twelve adults of the Cecropia silkworm were isolated, moistened with a film of insect Ringer containing streptomycin sulfate and phenylthiourea, and placed in the transparent, high pressure chamber described above under Methods. The strength of the heartbeat was studied in red and in white light in each of three CO/O_2 mixtures; namely, 100/1, 80/1, and 24/1, the oxygen tension in each case being recorded in Table I. Each preparation was observed for two hours.

Table I

Effects of carbon monoxide on the heartbeat of diapausing pupae and adult moths of Platysamia cecropia; light-reversibility of carbon monoxide inhibition

CO/O_2 Ratio	Oxygen tension (% atm.)	Amplitude of heartbeat			
		Diapausing pupae		Adult moths	
		Red light	White light	Red light	White light
100/1	5	+++	+++	++	+++
100/1	5	+++	+++	0	+++
100/1	5	++	+++	0	++
100/1	5			0	+++
100/1	5			+	+++
100/1	5			0	+++
100/1	5			0	+++
100/1	5			0	++
100/1	5			0	+++
80/1	5	+++	+++		
80/1	5	+++	+++		
80/1	5	+++	+++		
24/1	20	+++	+++	+	+++
24/1	20			+	+++
24/1	20			++	+++

The results are summarized in Table I. It will be observed that even the highest CO/O_2 ratios had little or no effect on the hearts of the diapausing pupae when the exposure was continued for two hours. By contrast, the adult hearts were strongly inhibited and this inhibition was fully or almost fully reversible in white light.

The light-reversibility in the case of adult hearts was especially dramatic when one region of heart was illuminated with white light and a closely adjacent region, with red light. The resumption of heartbeat in all cases was limited to the region

receiving the white light, the adjacent regions within the same heart remaining fully inhibited by carbon monoxide.

The experiments just considered for the Cecropia silkworm were repeated with similar results on adult hearts of the Polyphemus silkworm (*Telea polyphemus*).

The resistance of the pupal heart to high pressures of carbon monoxide was particularly impressive. However, it will be recalled that the heart at this stage possesses a substantial anaerobic metabolism (Harvey and Williams, 1958). Is it possible to account for the resistance to carbon monoxide on this basis? In order to test this possibility a series of four pupal hearts was isolated and exposed to a continuous flowing gas mixture containing 5% atm. oxygen and 95% atm. carbon monoxide (CO/O_2 ratio of 19/1). The carbon monoxide treatment was continued for 20 hours; *i.e.*, for a period greatly in excess of the anaerobic capacity of the pupal heart.

TABLE II

Effects on isolated hearts of prolonged exposure to a gas mixture containing 5 per cent oxygen in carbon monoxide (CO/O_2 ratio = 19/1)

Animal No.	Rate (beats/min.) and amplitude* of heartbeat								
	Air		Hours in CO/O_2 mixture						5 hours in air after 20 hours in CO/O_2 mixture
			2	3¾	5½	7	8½	20	
1	14 (3)	16 (3)	0 (0)	10 (3)	10 (3)	13 (3)	3 (3)	13 (3)	9 (2)
2	6 (3)	9 (3)	7 (3)	9 (3)	11 (3)	3 (3)	3 (3)	2 (3)	0 (0)
3	11 (3)	12 (3)	5 (3)	6 (3)	8 (3)	8 (3)	9 (3)	6 (3)	6 (3)
4	20 (3)	23 (3)	17 (3)	9 (3)	12 (3)	9 (3)	13 (3)	7 (3)	3 (3)
Average heartbeat index**	13	15	7.2	8.5	10	8.2	7.0	7.0	3.5

* Amplitudes scored and recorded in parentheses after rate.

** See Harvey and Williams (1958) for calculation of average heartbeat index.

The results are summarized in Table II. When examined in red light, all hearts were still beating at the end of 20 hours of treatment; indeed, the average heartbeat index was as high at this time as after two hours of exposure to carbon monoxide. Moreover, the performance of the hearts was not improved when they were transferred to air at this time. Therefore, this experiment, like the preceding one, reveals little clear-cut evidence of sensitivity of the pupal heart to carbon monoxide.

2. *The adult heart: further studies on the photo-reversal of CO-inhibition*

A series of five adult hearts was isolated and exposed to a CO/O_2 ratio of 100/1, the oxygen tension being 5% atm. and the carbon monoxide tension 5 atm. Examination under red light confirmed the prompt cessation of heartbeat. In three preparations the effect was fully reversed by white light; in two preparations partially reversed.

The inhibited hearts were exposed, in turn, to a series of five emission lines from

a mercury lamp (General Electric, AH5, 200 watts). The various lines were isolated by an appropriate series of Corning filters. Table III records the various combinations of filters and the relative energies of the corresponding emission lines; the latter were determined by the use of a thermopile in conjunction with a U. S. Bureau of Standards source.

In the results recorded in Table III no compensation has been made for the dissimilar relative energies. Notwithstanding this fact, it is sufficiently clear that the 436 and 579 mμ lines were maximally effective in reversing the carbon monoxide effect. These wave-lengths are in good proximity to the 450 and 589 mμ absorption maxima reported for the cytochrome oxidase-carbon monoxide complex of rat heart (Melnick, 1942). Of special interest is the low effectiveness of light at

TABLE III

Effectiveness of mercury emission lines in reversing the CO-inhibition of adult Cecropia and Polyphemus hearts*

Species	Wave-length	White	365 mμ	405 mμ	436 mμ	546 mμ	579 mμ
	Relative energy**		0.69	0.49	1.00	4.27	1.71
	Corning filter combination		No. 7380 No. 5860	No. 3060 No. 4308 No. 5970	No. 3389 No. 5113	No. 3484 No. 5120 No. 4303	No. 3480 No. 4305
Cecropia	Amplitude of heartbeat	+++	0	+	+++	+	+++
Cecropia		++	0	0	0	0	+
Cecropia		++	0	+	+++	+	+
Polyphemus		+++	0	0	++	0	+
Polyphemus		+++	0	0	+++	0	+++

* 5% atm. O_2 plus 500% atm. CO (ratio $CO/O_2 = 100/1$).
** Multiplication by 701 ergs/cm.²/sec. converts to absolute energy.

546 mμ notwithstanding its high relative energy. This emission and those at 365 and 405 mμ fall in regions of minimal absorption reported for the cytochrome oxidase-carbon monoxide complex.

The action spectrum—rough as it is—leaves little doubt that the effect of carbon monoxide on the adult heart depends on its combination with cytochrome oxidase. Therefore, cytochrome oxidase may confidently be identified as the terminal oxidase of the adult heart. However, the results, up to this point, suggest that cytochrome oxidase may not play this same role in the pupal heart. For this reason the pupal heart was studied in further detail.

3. *Oxygen tension and the pupal heartbeat*

Physiological activities which depend on the function of cytochrome oxidase may be recognized, not only in terms of their light-reversible inhibition by carbon monoxide, but also by the fact that they proceed at normal rates at very low oxygen tensions. This results from the extraordinary affinity of cytochrome oxidase for oxygen. According to Winzler (1941), the oxygen consumption of yeast becomes

maximal when the partial pressure of oxygen is only 0.25 to 2.5 mm. Hg. (0.03% to 0.3% atm.)—an affinity for oxygen which far exceeds that of any other known oxidase.

Having failed by the use of carbon monoxide to identify the terminal oxidase in the pupal heart, we sought to clarify the matter by studying the effects of oxygen tension on the function of the pupal heart. For this purpose a series of ten intact diapausing pupae, selected for pale pigmentation, was placed dorsal-side-up in a glass tube. Flowing mixtures of oxygen and nitrogen were then prepared and circulated through the tube, as described above under Section 4 of Methods. The heartbeat was observed within the intact pupae by the use of polarized light.

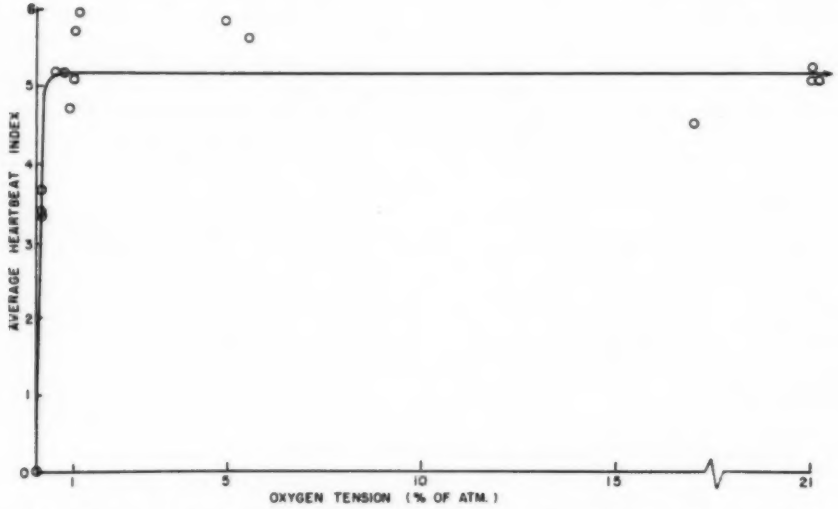


FIGURE 2. Effects of oxygen tension on the heartbeat of intact diapausing pupae of the Cecropia silkworm. The heartbeat is seen to be independent of oxygen at tensions at or above 0.5% atm. oxygen. In the determination of each datum, the exposure was continued for at least eight hours in order to compensate for the "anaerobic reserve."

In order to compensate for the anaerobic capacity of the pupal heart, the pupae were equilibrated with each gas mixture for eight hours prior to scoring the heartbeat. The same ten animals were used for the entire study, the tube being ventilated for eight hours with air before testing the next gas mixture.

The results are summarized and plotted in Figure 2 in terms of the average heartbeat index as a function of oxygen tension. It is extraordinary to observe that the heartbeat was independent of external oxygen tensions at or above 0.5% atm. Only when the pressure of oxygen was reduced below 0.5% atm. (4 mm. Hg) was there any detectable effect.

This result strongly argues that cytochrome oxidase is the terminal oxidase in the pupal heart, since no other oxidase is known to have the high affinity for oxygen implied in Figure 2.

4. CO-inhibition of the pupal heartbeat at low oxygen tensions

The finding that the hearts of intact diapausing pupae beat indefinitely at very low oxygen tensions paved the way for a more rigorous study of the effects of carbon monoxide than had been possible heretofore. Hearts could now be exposed to CO/O_2 ratios of 100/1 or higher. Moreover, the effects of carbon monoxide could be studied in the presence of very low oxygen tensions.

The following series of experiments had the objective of studying the heartbeat in the presence of varying pressures of carbon monoxide while holding the oxygen constant at a low pre-determined pressure. The constant oxygen pressures which were studied in turn were 0.18, 1.0, and 5.0% atm. The experimental set-up was essentially the same as that described under Section 3 above, except that the hearts were observed using polarized red light. Once again, ten intact diapausing pupae were used for the entire series of experiments.

TABLE IV

Effects of carbon monoxide at low oxygen tension on heartbeat of diapausing pupae

Ratio CO/O_2	5% atm. oxygen			1% atm. oxygen			0.18% oxygen		
	Average heartbeat index	Per cent inhibition	k(α)*	Average heartbeat index	Per cent inhibition	k(α)*	Average heartbeat index	Per cent inhibition	k(α)*
No CO	5.7			5.4			3.5		
3				5.4	0		1.6	53	2.4
10				3.5	35	19	0.8	76	3.2
18	5.6	2							
50				2.0	63	29	0.2	94	3.2
76	2.0	65	41						
99	1.3	78	28	0.9	83	20	0.1	97	3.0
555							0.0	100	
Average			34			23			2.9

* "Distribution constant" calculated from Warburg's partition equation (see text).

The first oxygen pressure to be studied in this manner was 0.18% atm. The pupae were equilibrated for eight hours with 0.18% atm. oxygen and 99.82% atm. of oxygen-free nitrogen. While holding the oxygen concentration constant, a certain percentage of carbon monoxide was then added to the flowing mixture of oxygen and nitrogen; namely, 0.54, 1.8, 9.0, 18.0, and 99.82% atm. carbon monoxide. The animals were equilibrated with each mixture for eight hours before recording the heartbeat. And before testing successive carbon monoxide mixtures, the system was ventilated for eight hours with 0.18% atm. oxygen and 99.82% atm. nitrogen.

The results are recorded in Table IV and the lower curve in Figure 3. It will be observed that in the absence of carbon monoxide the heartbeat index was already depressed by the low oxygen tension *per se*. As increasing percentages of carbon monoxide were added, one witnesses a further depression in heartbeat index attributable to carbon monoxide. Finally, at the low oxygen tension under consideration (0.18% atm.), the heartbeat is totally blocked at a CO/O_2 ratio of 555/1.

In like manner the experiment was repeated utilizing oxygen at the constant pressure of 1% atm. The results recorded in Table IV and Figure 3 show no depression attributable to the low oxygen pressure *per se*. However, as carbon monoxide is added, the heartbeat index begins to decline and continues to do so until one establishes a CO/O_2 ratio of 99/1—the highest ratio attainable at a total pressure of one atmosphere.

Finally, the experiment was repeated using oxygen at the constant pressure of 5% atm. A CO/O_2 ratio of 19/1 was the highest ratio attainable at one atmosphere total pressure. Therefore, the animals at this point were transferred to the transparent pressure chamber and the experiment continued at positive pressures up to

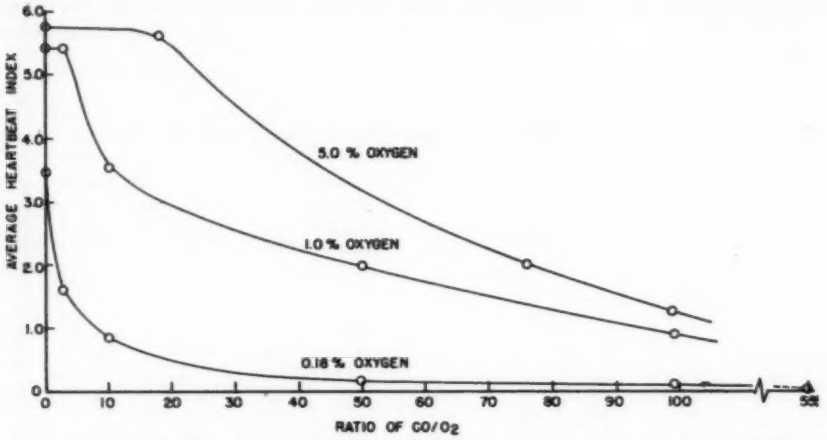


FIGURE 3. The effects of increasing concentrations of carbon monoxide on the heartbeat observed in intact diapausing Cecropia pupae. In the determination of each datum the experiment was continued for at least eight hours in order to compensate for the anaerobic reserve. The effects of carbon monoxide are dictated, not only by the CO/O_2 ratio, but also by the absolute tension of oxygen.

five atmospheres. The results summarized in Table IV and Figure 3 show no inhibition attributable to the low oxygen pressure *per se*. Moreover, the carbon monoxide inhibition now becomes apparent only at high CO/O_2 ratios; *i.e.*, in excess of 18/1.

5. *Photoreversal of the CO-inhibition of the pupal heart*

By the same technique described in Section 4, nine diapausing pupae were equilibrated for eight hours with a flowing mixture of 1% atm. oxygen and 99% atm. carbon monoxide (CO/O_2 ratio of 99/1). The hearts were then examined in polarized red light and the heartbeat index calculated. All hearts were strongly inhibited, and four of the nine were not beating. Each heart, in turn, was then illuminated with a pair of focussed beams of intense white light, and the effects scored over a period of five minutes. The results recorded in Table V reveal a marked reversal of the carbon monoxide inhibition by white light.

As a control for the preceding experiment, the same group of animals was equilibrated for eight hours with a flowing mixture of 1% atm. oxygen and 99% atm. nitrogen. The heartbeat index was then recorded in red light and white light, as just described. No inhibition was observed either in red or in white light (Table V).

TABLE V

Light reversibility of the CO-inhibition of pupal hearts (within intact animal)

Animal No.	Rate (beats/min.) and amplitude* of heartbeat			
	99% CO 1% O_2 Red light	99% CO 1% O_2 White light	99% N_2 1% O_2 Red light	99% N_2 1% O_2 White light
1	0 (0)	6 (3)	3 (3)	4 (3)
2	3 (1)	7 (3)	11 (3)	16 (3)
3	0 (0)	14 (3)	4 (3)	7 (3)
4	1 (1)	3 (3)	3 (3)	3 (3)
5	0 (0)	9 (3)	3 (3)	13 (3)
6	3 (2)	3 (3)	4 (3)	4 (3)
7	0 (0)	4 (3)	8 (3)	6 (3)
8	2 (1)	9 (3)	3 (3)	2 (3)
9	1 (2)	2 (3)	5 (3)	4 (3)
Average heartbeat index**	0.44	5.7	4.6	5.9

* Amplitudes scored and recorded in parentheses after rate.

** See Harvey and Williams (1958) for calculation of average heartbeat index.

6. *Lethal effects of carbon monoxide at low oxygen pressure*

In the experimental results described above, normal heartbeat was maintained in nitrogen containing 1% atm. oxygen. By contrast, the heart was strongly inhibited in carbon monoxide containing 1% atm. oxygen. These results lead to the prediction that carbon monoxide should be a lethal agent for diapausing pupae when administered in combination with low oxygen pressure.

Five diapausing pupae were placed in each of two 3.5-liter Lucite pressure chambers along with a small dish of 10 per cent potassium hydroxide to absorb carbon dioxide. One chamber was flushed daily with ten volumes of a gas mixture containing 1% atm. oxygen in nitrogen; the other, with a mixture of 1% atm. oxygen in carbon monoxide. The experiment was continued for 14 days. The chambers were then opened and the animals examined. All of the control animals in nitrogen were lively and normal; all the experimental animals in carbon monoxide were flaccid and dead.

DISCUSSION

1. *The heartbeat of the adult moth*

The heartbeat of the adult moth is strongly inhibited by cyanide in that HCN, in a concentration of less than 10^{-5} *M*, inhibits the heartbeat by 50 per cent (Harvey

and Williams, 1958). This finding is strong evidence that the heartbeat in the adult moth depends on the function of cytochrome oxidase.

In the present study the isolated adult heart was inhibited when the ratio of carbon monoxide to oxygen was 24/1 in the surrounding gas phase. This inhibition was promptly reversed by light. Moreover, the effectiveness of the light coincides with the absorption maxima of the cytochrome oxidase-CO complex. Therefore, the terminal oxidase of the adult heart may confidently be identified as cytochrome oxidase. This conclusion is consistent with the finding that the adult heart contains a high concentration of the several cytochromes including cytochrome oxidase (Shappirio and Williams, 1957a, 1957b).

2. The heartbeat of the diapausing pupa

As described in Sections 1 and 4 of the Results, it is easy to get the impression that the pupal heart differs from the adult heart in being insensitive to carbon monoxide. Even when the ratio of carbon monoxide to oxygen is raised as high as 19/1, the pupal heart continues to beat normally, provided that at least 5% atm. oxygen is present.

In the analogous case of the apparent insensitivity of the pupal heart to cyanide (Harvey and Williams, 1958), a satisfactory explanation was found to be the anaerobic capacity of the heart at this particular stage. However, this same explanation does not suffice to explain the insensitivity of the pupal heart to carbon monoxide. Thus, as we have seen (Table II), the pupal heart beats indefinitely in mixtures of carbon monoxide and oxygen (19/1), whereas it comes to a standstill after several hours in oxygen-free nitrogen. Therefore, on the basis of results of this type, one might be persuaded that the pupal heart fails to make use of CO-sensitive cytochrome oxidase, notwithstanding the presence in the pupal heart of a substantial titer of this same enzyme.

The present study makes clear that this conclusion is erroneous. It is our present purpose to show how the relatively high concentration of cytochrome oxidase in the pupal heart serves to camouflage the true sensitivity of the pupal heart to carbon monoxide.

3. Pupal heartbeat at low oxygen tensions

The resistance of the pupal heart to carbon monoxide can be accounted for most easily by first considering the insensitivity of the same heart to low oxygen tensions. The two phenomena, as we shall see, have a very close connection.

As illustrated in Figure 2, the heart of the diapausing pupa continues to beat normally for an indefinite period when the intact pupa is exposed to oxygen tensions as low as 0.5% atm. In experiments which are sufficiently prolonged to compensate for the anaerobic reserve of the pupal heart, one finds that the heartbeat is blocked in the total absence of oxygen and significantly depressed at external tensions lower than 0.5% atm. This implies that, after the exhaustion of its anaerobic reserve, the pupal heart is "micro-aerophilic." Oxygen is required, but a very low tension suffices. The fact that an external tension as low as 0.5% atm. sustains the normal heartbeat is especially impressive when one recalls that a tension of 0.5% atm. oxygen outside the insect corresponds to a still lower tension in the heart muscle itself.

In our opinion the micro-aerophilic character of the pupal heart can be accounted for in terms of the presence in the pupal heart of a substantial titer of cytochrome oxidase in conjunction with only a trace of its substrate *c*. Consider the situation in Figure 4A descriptive of the circumstances in the heart of the diapausing pupa. Here the trace of cytochrome *c* is diagrammed as passing electrons to the oxidized heme (+ + +) of cytochrome oxidase. The latter is present in the diapausing heart in great excess over cytochrome *c*. Provided that the trace of *c* has free access to the pool of oxidase, a steady-state will be established in which the reduction of oxidase is more than counterbalanced by the oxidation of oxidase by molecular oxygen. Therefore, the vast majority of cytochrome oxidase exists in the oxidized state. As diagrammed in Figure 5A, one can lower the oxygen tension, thereby establishing a new steady-state in which half of the oxidase is in the oxidized form and half in the reduced form. The respiration, as signalled by the rate of water formation, remains unimpaired.

The presence of the great excess of cytochrome oxidase has a further influence on the kinetics of this steady state. At very low oxygen tensions, most of the oxidase will be present in the reduced (+ +) state. This increases the concentration of reduced oxidase, the target of molecular oxygen, and permits a still lower oxygen tension to suffice. The excess oxidase acts, in effect, as a "buffer" against very low oxygen tensions. It is important to note that under all these circumstances the rate of electron transmission and of oxygen consumption (represented by the block marked H_2O in the diagrams) remain constant and independent of oxygen tension.

The set of circumstances, diagrammed in Figure 6A, considers the situation when the oxygen tension is so low as to limit the respiration. Almost all of the oxidase is now reduced. Finally, in a completely anaerobic situation, all the oxidase becomes reduced and the respiration is totally blocked at this point.

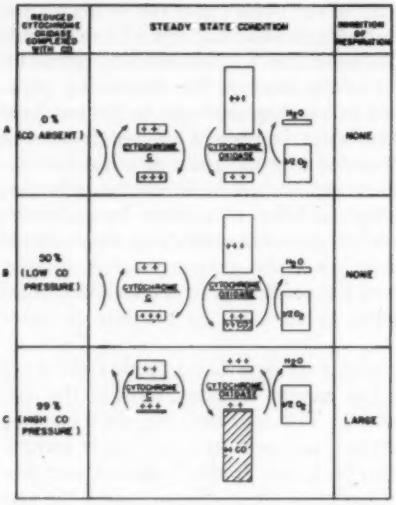
For these several reasons we can understand the reason why the pupal heart continues to beat normally in the presence of oxygen tensions as low as 0.5% atm. The explanation is found to lie in the disproportionately high concentration of cytochrome oxidase with respect to cytochrome *c*—the precise combination that characterizes the heart of the diapausing pupa (Shappirio and Williams, 1957a, 1957b). The surplus oxidase is "money in the bank" which can be drawn upon for transactions at low oxygen pressures.

4. *A theoretical consideration of CO-inhibition*

As Goddard (1947) points out, a mathematical treatment of the kinetics of the CO-inhibition of cytochrome oxidase requires so many simplifying assumptions that the formulation is scarcely descriptive of any real experiment. Indeed, we are unable to find in a search of the literature any satisfactory non-mathematical treatment. Consequently, we have been forced to develop a semi-diagrammatic presentation of the facts. Though descriptive of the experiments on the silkworm heart, we believe that the formulation will be pertinent to analogous studies.

A. *CO-inhibition at normal oxygen pressures*

The crux of the matter is that carbon monoxide combines only with the reduced form of cytochrome oxidase, the CO/O_2 ratio dictating the percentage of reduced oxidase which is complexed and inactivated. By virtue of the low concentration of



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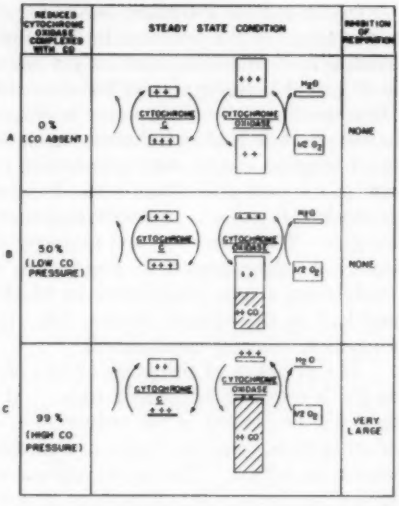

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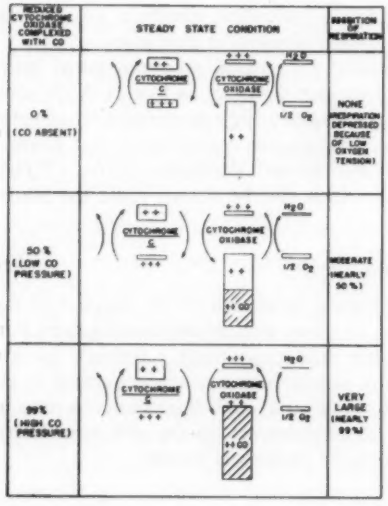

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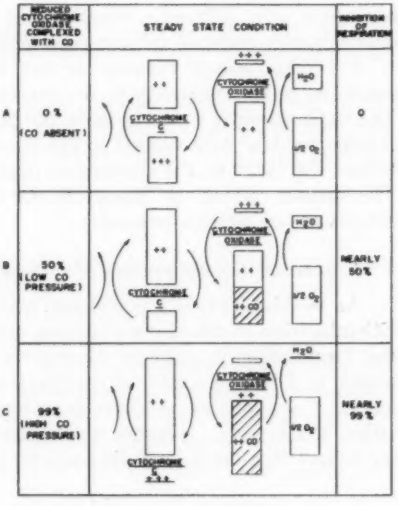

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FIGURE 4. CO-inhibition of the respiration of diapausing Cecropia pupae. Diagram of the steady-state condition of the electron transport system when the oxygen tension is not limiting respiration (5% atm. oxygen or above).

cytochrome *c* a correspondingly low concentration of reduced oxidase exists in the normal pupal heart at ordinary oxygen tensions (Fig. 4A). Under this circumstance carbon monoxide finds a limited target within the diapausing heart. The addition of sufficient carbon monoxide to complex, say, 50 per cent of the reduced oxidase transiently slows the rate of oxidation of reduced cytochrome oxidase. This causes additional oxidase to accumulate in the reduced form until the amount of reduced oxidase is twice that present in the absence of carbon monoxide (Fig. 4B). Although 50 per cent continues to be complexed by carbon monoxide, in the new steady-state the amount of uncomplexed reduced oxidase becomes the same as it had been in the total absence of carbon monoxide. Consequently, the respiration is uninhibited.

At very high pressures of carbon monoxide sufficient to complex, say, 99 per cent of reduced oxidase, the reserve of oxidase becomes limiting and the system can no longer undergo the necessary degree of internal compensation (Fig. 4C). Therefore, the rates of electron transfer, oxygen consumption, and water formation are slowed down.

B. *CO-inhibition at low oxygen pressures*

As we have just seen, the concentration of reduced cytochrome oxidase and the sensitivity to carbon monoxide can be enhanced experimentally by lowering the oxygen tension. Let us consider the hypothetical case where the oxygen pressure is lowered until 50 per cent of the oxidase is in the reduced form (Fig. 5A). The rate of oxygen consumption and water formation remains the same as at higher oxygen pressures.

If one now adds enough carbon monoxide to complex 50 per cent of the reduced oxidase, a new steady-state results in which nearly all the oxidase shifts to the reduced condition (Fig. 5B). Whether the respiration will be inhibited will be determined by whether sufficient oxidase is present to supply the necessary degree of compensation. In the case considered in Figure 5B, this condition is fulfilled and the rate of water formation is diagrammed as uninhibited. However, as shown in Figure 5C, the compensatory mechanism breaks down if the pressure of carbon monoxide is further increased. A strong inhibition of respiration and of water formation is then observed.

C. *CO-inhibition at very low oxygen pressures*

Attention is finally directed to the set of circumstances diagrammed in Figure 6. The oxygen pressure at the outset is reduced to a very low level (0.18% atm.) so that the respiration is already inhibited and most of the cytochrome oxidase is present in the reduced form (Fig. 6A). The reserves of oxidized

FIGURE 5. CO-inhibition of the respiration of diapausing Cecropia pupae. Diagram of the electron transport system when the oxygen tension is low but not limiting respiration (1% atm. oxygen).

FIGURE 6. CO-inhibition of the respiration of diapausing Cecropia pupae. Diagram of the electron transport system when the oxygen tension is limiting respiration (0.18% atm. oxygen).

FIGURE 7. CO-inhibition of the respiration of developing adults of the Cecropia silkworm. Diagram of the steady-state condition of the electron transport system when the oxygen tension is not limiting the respiration. The resynthesis of cytochrome *c* enhances the metabolism and the sensitivity to carbon monoxide.

oxidase have already been exhausted and the system is immediately sensitive to low pressures of carbon monoxide (Figs. 6B and 6C).

5. *Carbon monoxide and the pupal heart*

Under experimental conditions the pupal heart is found to respond to carbon monoxide in accordance with theory. In Figure 8 the experimental results of

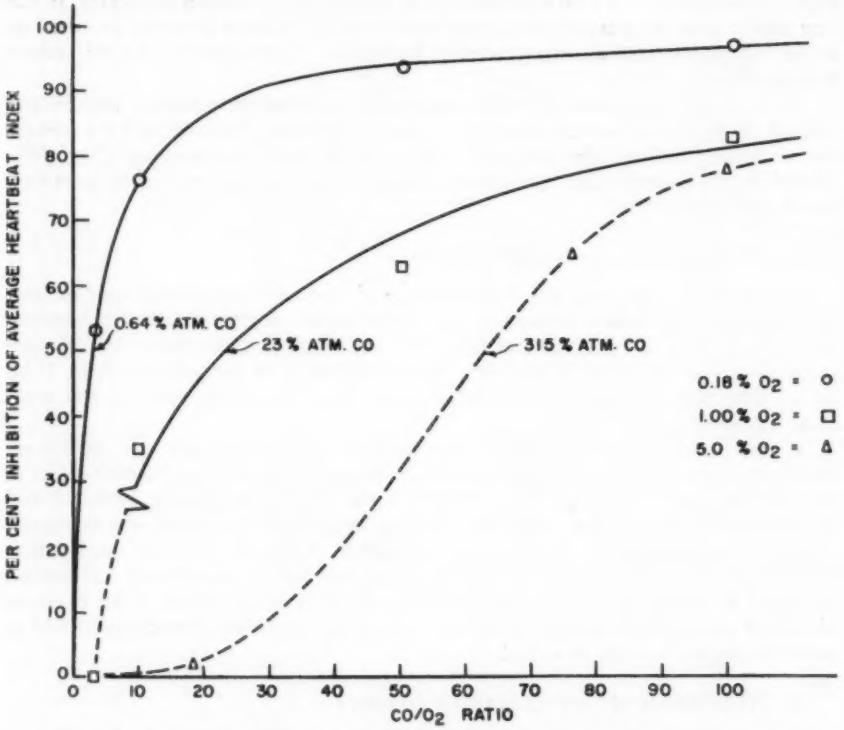

FIGURE 8. Per cent inhibition of the heartbeat of diapausing Cecropia pupae as a function of the CO/O_2 ratio. The inhibition is determined, not only by the CO/O_2 ratio, but also by the absolute tension of oxygen. The uppermost curve has been drawn in accordance with the Warburg equation.

Table IV and Figure 3 have been brought together and plotted on a scale in which the per cent inhibition of heartbeat is considered as a function of the CO/O_2 ratio at each of three oxygen pressures.

At the very low oxygen pressure of 0.18% atm., the heart is inhibited by any finite pressure of carbon monoxide and 50 per cent inhibited by carbon monoxide at a pressure of only 0.64% atm. (5 mm. Hg). When the oxygen pressure is raised to 1% atm., then the carbon monoxide pressure must be increased to 23%

atm. to achieve 50 per cent inhibition. Finally, at the still higher oxygen pressure of 5% atm., 50 per cent inhibition is brought about only by a very high pressure of carbon monoxide (315% atm.).

6. The critical effects of cytochrome c

The analysis, up to this point, has focussed attention on the oxidation of cytochrome oxidase by molecular oxygen. In a given system at low oxygen pressures, the pressure of oxygen dictates the partition of oxidase between oxidized and reduced states; this partition, in turn, is found to condition the sensitivity to carbon monoxide. An examination of Figure 7 will make clear that this same partition can be influenced at any specific oxygen pressure by varying the rate of reduction of cytochrome oxidase. This is achieved by increasing the concentration of reduced cytochrome *c*.

It is precisely this circumstance which supervenes at the initiation of adult development. As Shappirio and Williams (1957a, 1957b) have shown, a rapid synthesis of cytochrome *c* occurs at this time. The metabolism is enhanced and now shows a definite sensitivity to carbon monoxide even at high oxygen pressures (Schneiderman and Williams, 1954a). This result is intelligible in terms of the discussion set forth above.

Shappirio and Williams (1957b) have been able to duplicate this same phenomenon *in vitro* by the addition of extrinsic cytochrome *c* to washed homogenates of diapausing pupal tissues (using DPNH as substrate). The metabolism of the homogenate increases markedly and shows a substantial sensitivity to inhibitors of cytochrome oxidase.

7. Warburg's equation for CO-inhibition

In the hypothetical situation where all the cytochrome oxidase is in the reduced condition, then, as Warburg (1927) points out, one can formulate a simple stoichiometric relation between the inhibition of respiration and the CO/O_2 ratio.

$$\frac{\text{Respiration uninhibited by CO}}{\text{Respiration inhibited by CO}} = K\,\frac{O_2}{CO}$$

In practice it is difficult or impossible to establish a steady-state in which all the oxidase is reduced. Biochemists have routinely attempted to satisfy this requirement by the addition to *in vitro* systems of a large excess of substrates and cytochrome *c*.

The present investigation directs attention to a far simpler solution of the problem which is applicable, not only to *in vitro* systems, but also to intact organisms. This technique, as we have seen, is to favor the reduction of oxidase by working at very low oxygen pressures. Under this circumstance the quantitative aspects of the carbon monoxide inhibition are in precise agreement with Warburg's formulation. This fact is evident in Figure 8 where the uppermost curve is a theoretical curve constructed according to the Warburg equation, the constant K being 3. This low value implies that carbon monoxide has a higher affinity for reduced cytochrome oxidase than is customarily assumed.

8. The terminal oxidase during metamorphosis

We are persuaded by the argument outlined above that the heartbeat of the diapausing pupa is sustained by the CO-sensitive cytochrome oxidase. Confirmation of this view is found in the demonstration that the CO-inhibition of the pupal heartbeat is promptly reversed by light (Table V). The reason that the pupal heart appears to be insensitive to carbon monoxide is that the true sensitivity is camouflaged by the great excess of cytochrome oxidase that is present. In our opinion many additional instances of so-called CO-insensitive respiration reported in the literature will be found to have a similar basis.

In the earlier studies we have interpreted the CO-resistance of the diapausing pupa to signal the presence of a CO-insensitive oxidase. However, it is worth recalling that the non-muscular tissues of the diapausing pupa also contain cytochrome oxidase in great excess over cytochrome *c* (Shappirio and Williams, 1957a and b). Therefore, if cytochrome oxidase can give the false impression of CO-insensitivity in the heart, there is no *a priori* reason why it cannot do so in the non-muscular tissues. In this connection it is of interest that the pupa as a whole is killed by exposure to carbon monoxide at low oxygen pressures; *i.e.*, under conditions where the excess of cytochrome oxidase is eliminated from the field of action (Section 6 of Results).

The present study of the heart does not permit a clear decision as to the role of cytochrome oxidase in the pupa as a whole. Most fortunately, however, this particular matter has simultaneously been studied in an independent investigation by Kurland and Schneiderman, and will be considered in detail in a forthcoming publication.

Summary

1. The heartbeat of the adult Cecropia moth is inhibited by suitable pressures of carbon monoxide, and this inhibition is reversed by light.

2. The wave-lengths which are maximally effective in reversing the CO-inhibition are in good agreement with the absorption maxima of the CO-cytochrome oxidase complex.

3. Therefore, the terminal oxidase of the adult heart may be identified as cytochrome oxidase.

4. The situation is much more complex in the case of the diapausing pupa. The latter shows a considerable capacity for anaerobic metabolism, and its heart can beat for several hours in the total absence of oxygen. Moreover, normal heartbeat continues indefinitely in the presence of oxygen pressures as low as 0.5% atm.

5. After exhaustion of the "anaerobic reserve," the pupal heart may be said to be "micro-aerophilic." Oxygen is required but a remarkably low tension suffices.

6. At ordinary oxygen pressures such as that in air, it is difficult or impossible to demonstrate any sensitivity of the pupal heart to carbon monoxide. However, when the oxygen tension is decreased to very low levels, the heartbeat shows a clear sensitivity to carbon monoxide. Thus, at the low oxygen tension of 0.18% atm., the pupal heartbeat is inhibited 50 per cent by carbon monoxide at a pressure of only 5 mm. Hg.

7. Under these circumstances, the inhibition of the pupal heartbeat is light-reversible.

8. The actions of oxygen and carbon monoxide are considered in detail. The terminal oxidase of the pupal heart is found to be cytochrome oxidase.

9. The resistance of the pupal heart to carbon monoxide and to low oxygen pressures can be accounted for in terms of the presence in the pupal heart of a great excess of cytochrome oxidase relative to cytochrome *c*.

LITERATURE CITED

EPHRUSSI, B., AND G. W. BEADLE, 1936. A technique of transplantation for *Drosophila*. *Amer. Nat.*, **52**: 218–225.

GODDARD, D. R., 1947. Respiratory enzymes. *In:* Physical Chemistry of Cells and Tissues, (R. Höber, editor), London. J. and A. Churchill, Ltd.: 397–420.

HARVEY, W. R., AND C. M. WILLIAMS, 1958. Physiology of insect diapause. XI. Cyanide-sensitivity of the heartbeat of the Cecropia silkworm, with special reference to the anaerobic capacity of the heart. *Biol. Bull.*, **114**: 23–35.

HILL, R., AND E. F. HARTREE, 1953. Hematin compounds in plants. *Ann. Rev. Plant Physiol.*, **4**: 115–150.

MELNICK, J. L., 1942. The photochemical spectrum of cytochrome oxidase. *J. Biol. Chem.*, **146**: 385–390.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1954a. The physiology of insect diapause. VIII. Qualitative changes in the metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.*, **106**: 210–229.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1954b. The physiology of insect diapause. IX. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bull.*, **106**: 238–252.

SCHNEIDERMAN, H. A., AND N. FEDER, 1954. A respirometer for metabolic studies at high gaseous pressures. *Biol. Bull.*, **106**: 230–237.

SHAPPIRIO, D. G., AND C. M. WILLIAMS, 1957a. The cytochrome system of the Cecropia silkworm. I. Spectroscopic studies of individual tissues. *Proc. Roy. Soc. London, Ser. B*, **147**: 218–232.

SHAPPIRIO, D. G., AND C. M. WILLIAMS, 1957b. The cytochrome system of the Cecropia silkworm. II. Spectrophotometric studies of oxidative enzyme systems in the wing epithelium. *Proc. Roy. Soc. London, Ser. B*, **147**: 233–246.

WARBURG, O., 1927. Über die Wirkung von Kohlenoxyd und Stickoxyd auf Atmung und Gärung. *Biochem. Zeitschr.*, **189**: 354–380.

WARBURG, O., 1949. Heavy metal prosthetic groups and enzyme action. Oxford at the Clarendon Press. pp. 230.

WILLIAMS, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.* **90**: 234–243.

WINZLER, R. J., 1941. The respiration of bakers' yeast at low oxygen tension. *J. Cell. Comp. Physiol.*, **17**: 263–276.

NOTES ON THE BIOLOGY OF THE FIVE-LUNULED SAND DOLLAR

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The five-lunuled sand dollar, *Mellita quinquiesperforata* (Leske, 1778), is abundant on the sand flats at Beaufort, North Carolina, where the following observations were made during a short stay in August, 1957, at the Duke University Marine Laboratory.

Burrowing. The animal lives in shallow water in a horizontal position, usually completely covered by a thin layer of sand although sometimes the posterior lunule remains visible. Specimens observed were motionless although the animal can progress under the sand. On being removed and placed on top of the sand under water it promptly burrows under again. The statements about its method of burrowing in Pearse, Humm and Wharton (1942) are totally at variance with my observations and appear wholly erroneous. They say that in burrowing the animal moves directly downward, waving its spines and tube feet so as to move sand from beneath the test to the upper surface, and does not progress anteriorly as it descends. My observations on animals in their natural habitat agree with those of Kenk (1944) on the closely related *Mellita lata* at Puerto Rico. Unlike regular urchins, sand dollars and other irregular urchins are polarized with definite anterior and posterior ends and can move only in the anterior direction, that opposite the posterior lunule in the present species. When placed on the sand under water in its natural habitat *Mellita* promptly begins to bury itself. It does this by simply advancing into the sand in a horizontal or slightly oblique orientation, using its ordinary method of locomotion by waves of movement along the spines of the oral surface. Kenk gives a succession of photographs showing *Mellita* advancing into the sand, which rapidly covers it. I did not time the reaction but only a couple of minutes is required for the five-lunuled species to cover itself with a thin layer of sand whereupon it comes to a halt. It positively does not move downward, does not remove sand from beneath it, and does progress forward. It is improbable that the tube feet play any role in locomotion as they are small and feeble and of limited distribution.

Lunules. Contrary to the statement of the Berrills (1957), in *Mellita* sand positively does not pass through the lunules from the oral to the aboral surface during the burrowing process. To the contrary as the animal advances into the sand, sand falls through the lunules to the oral surface, thus if anything retarding the burying process. It is known for a Japanese species (Ikeda, 1941) that the lunules are important in burrowing and righting but this is definitely not the case in *Mellita.* The further suggestion of the Berrills that the lunules strengthen the test by fusing the oral and aboral surface is extremely improbable because the two surfaces of the test are so much connected by interior

calcareous columns that little space remains for the viscera of the animal. The function of the lunules in the five-lunuled sand dollar remains enigmatical. When the animal is placed in a bowl of sea water without sand the spines along the lunules are seen in constant activity but no current could be detected passing through the lunules. If the animal is lifted out of water these spines bend so as to close the lunules. If the animal is placed in a clean bowl of sea water in order to view the oral surface and the aboral surface covered with sand, to make the animal more comfortable, so to speak, sand falls through the lunules as the animal peregrinates around the bowl and covers the bottom, preventing a view of the oral surface. It is difficult to ascribe any value in nature to this behavior.

Podia. Very little information is available in the literature concerning the distribution of the podia or tube feet of sand dollars, apart from the specialized feet of the aboral petaloids. About the only detailed reports are those of Gregory (1911) and Parker and Van Alstyne (1932) for the New England sand dollar, *Echinarachnius parma;* here the podia are said to occur abundantly along the radii of both oral and aboral surfaces and also around the entire margin. Unlike the situation in regular urchins where each podium passes through two holes in the test, in sand dollars there is one hole for each podium. It does not seem to be generally realized that the distribution of the podia in any sand dollar can be determined by examining the cleaned test under low magnification whereupon the small holes through which the podia emerge are seen. Kenk (1944) stated that in *Mellita lata* podia occur along the margins of the lunules and among the spines of the entire circumference as well as along the ambulacral furrows of the oral surface. In the five-lunuled *Mellita* repeated and careful examinations of cleaned tests revealed the small holes for the podia *only* along the ambulacral furrows of the oral surface. They are definitely absent from the margins of the lunules and the edge of the test. These furrows lack spine tubercles that are present everywhere else on the test. Despite repeated efforts I was never able to see the podia on living animals turned oral side up in a dish of sea water under magnification. Presumably under such abnormal conditions the podia are retracted. The pedicellariae are easily seen, constantly jerking about, and may have been mistaken for podia by some observers. They are very numerous on the areas between the ambulacral furrows and this is rather curious because Mortensen (1948) declares that he could rarely find pedicellariae in *Mellita.*

Feeding. My main purpose was to observe the feeding process but in this I failed signally. The only observations on the feeding of sand dollars are those of the MacGinities (1949). They state that the cilia of the aboral spine bases create currents that carry food particles entrapped in mucus to the posterior edge where they are passed to the oral side and proceed along the ambulacral furrows to the mouth. In reply to inquiries, Dr. MacGinitie kindly stated that the species used was *Dendraster excentricus* (or a variant thereof), that juveniles are best for observations of this kind, and that they must be acclimated to laboratory conditions for a week or more. Inspection of the cleaned tests of *Dendraster excentricus* showed that the podial pores are limited to the ambulacral furrows and that these furrows are devoid of spines. MacGinitie does not seem to have observed the podia and when questioned as to the mechanism of movement of the mucous-coated food particles along the ambulacral furrows

replied that it is ciliary. I am of the opinion that the podia must be the chief mechanism of food movement along the furrows as they seem to have no other function, not being concerned in locomotion.

My observations were made on freshly collected specimens placed in shallow dishes of sea water without sand. Carmine grains and bits of crushed plankton (kindly supplied by Dr. Frank Maturo), untreated or stained with neutral red or mixed with carmine grains, were placed on various sites on the aboral or oral surfaces, including the ambulacral furrows, and observed over a long period. No trace of movement of the particles was seen in any location nor was there any evidence of ciliary currents anywhere. This negative result is probably to be attributed to the abnormal conditions under which the animals were kept. Unfortunately it did not occur to me to examine the spines microscopically for the presence of cilia. A morsel of plankton placed among the marginal spines caused commotion among them; they converged on the morsel and manipulated it for a long time but it was never moved to the slightest extent from the site on which it had been placed. Evidently to determine the mode of feeding of *Mellita* will require time, patience and ingenuity. It is difficult to think of any method by which these animals can be kept in normal conditions that at the same time will permit a view of the oral surface.

The digestive tract of one specimen was removed and the contents examined under the compound microscope. The intestine contained no sand grains nor any coarse material nor any remains of larger organisms. The contents consisted entirely of multitudes of minute naked organisms, all seemingly of one kind, apparently nannoplankton.

LITERATURE CITED

Berrill, N. J., and J. Berrill., 1957. 1001 Questions Answered About the Seashore. Dodd, Mead, and Company, New York; xii + 305 pp.

Gregory, Emily, 1911. Observations on the water-vascular system of *Echinarachnius parma*. *Zool. Anz.*, **38**: 323–326.

Ikeda, M., 1941. Function of the lunules of *Astriclypeus* as observed in the righting movement (Echinoidea). *Annot. Zool. Japon.*, **20**: 78–82.

Kenk, R., 1944. Ecological observations on two Puerto Rico echinoderms, *Mellita lata* and *Astropecten marginatus*. *Biol. Bull.*, **87**: 177–187.

MacGinitie, G. E., and N. MacGinitie., 1949. Natural History of Marine Animals. McGraw-Hill Book Company, New York; xii + 473 pp.

Mortensen, Th., 1948. A monograph of the Echinoidea, vol. IV, part 2. C. A. Reitzel, Copenhagen; 471 pp.

Parker, G. H., and M. Van Alstyne, 1932. Locomotor organs of *Echinarachnius parma*. *Biol. Bull.*, **62**: 195–200.

Pearse, A. S., H. J. Humm and G. W. Wharton, 1942. Ecology of sand beaches at Beaufort, N. C. *Ecol. Monogr.*, **12**: 137–190.

SOME ASPECTS OF BEHAVIOR OF OYSTERS AT DIFFERENT TEMPERATURES

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The oyster, *Crassostrea virginica* Gmelin, is a sedentary mollusk which must pump definite quantities of water through its gills, to obtain the food and oxygen necessary for its existence, and to get rid of the waste products. The stream of water created by a pumping oyster can easily be seen if the latter is kept in a shallow tray in water just deep enough to cover it. Even casual observations will show that the quantity of water pumped by an oyster changes from time to time, and that different oysters may pump at different rates.

In keeping oysters under laboratory conditions it is, obviously, of advantage to know the approximate quantity of water needed for their normal existence. The same information is of practical use to oyster cultivators in deciding the number of oysters that can be planted in a certain area to achieve the best growth. Furthermore, because of the recent progress in the artificial propagation of bivalves (Loosanoff and Davis, 1950; Loosanoff, 1954), which is now leading to mass production of clams and oysters under hatchery conditions, and because of the recent interest in the utilization for shellfish culture of small, salt water ponds, where annual fluctuations in temperatures may be great, extending in some regions from nearly 0.0° to 32.0° C. or even higher, a more complete knowledge of the behavior of the mollusks within this temperature range is needed. Finally, the differences in the rates of water pumping at the same temperatures by oysters from different geographic areas may be used as the criterion to ascertain the existence of different physiological races.

Studies of various aspects of filtration of the filter-feeding invertebrates, including oysters and closely related mollusks, have been made by many investigators. Comprehensive reviews of several hundred articles on these subjects were recently offered by Verwey (1952) and Jørgensen (1955); therefore, only those dealing directly with rates of water pumping by bivalves, especially oysters, will be referred to here.

The first comprehensive studies of the rate of pumping of the American oyster, *C. virginica,* at different temperatures were made by Galtsoff (1928a, 1928b). He used two methods. The first, the so-called "tank method," was designed primarily to collect the water after it had passed through the gills. The second or "carmine method" was devised to measure the rate of movement of the column of water flowing from the exhalant chamber through a glass tube. Galtsoff concluded that the maximum flow of water produced by an adult oyster, three to four inches in length, was 3.9 liters per hour at a temperature of 25.0° C. Galtsoff's conclusions were challenged by Nelson (1935, 1938) who considered these figures too low and thought that the methods used by Galtsoff unfavorably affected the experimental

animals. Nelson, therefore, believed that Galtsoff's data did not truly present the normal activities of oysters. Nelson (1935) also reported that, instead of the 3.9 liters per hour indicated by Galtsoff as the maximum quantity of water that can be pumped by an oyster, some of the animals in his experiments pumped water at the rate of approximately 26.0 liters per hour. In our own work, we have recorded pumping rates of individual oysters as high as 34.0 liters per hour, *i.e.*, about ten times greater than Galtsoff's maximum (Loosanoff and Nomejko, 1946).

Most of Nelson's observations, as well as ours and those of several other investigators, were made, however, while studying various aspects of the physiological behavior of oysters and not during the studies devoted primarily to the evaluation of the effects of temperature upon their pumping rate. Since the need of more complete information on this subject still existed, the experiments discussed in this paper were conducted.

I wish to express my appreciation to Charles A. Nomejko for tabulating some of the data and for preparing photographs of the kymograph records, to Barbara J. Myers for the statistical treatment of the data used in this article and to Rita S. Riccio for her help in preparing the manuscript.

Methods

Only Long Island Sound oysters were used in these studies. For the sake of uniformity, they were selected to approximate the following standards: length—between 100.0 and 110.0 mm.; width—between 80.0 and 85.0 mm.; depth—between 30.0 and 35.0 mm.; and volume—between 85.0 and 100.0 cc. Before the oysters were used they were conditioned for several days at the temperature to be employed in the experiment.

The method for measuring the rate of flow of water through the gills of the oysters was based on the suggestions and apparatus of Moore (1910), Galtsoff (1926) and Nelson (1936), and was fully described in our article on feeding of oysters in relation to different concentrations of micro-organisms (Loosanoff and Engle, 1947). Here it is sufficient to mention that the so-called "rubber apron" method interfered in no way with the normal activities of the oysters and allowed us to collect and measure accurately the quantities of water pumped by them. Both the rate of pumping and the shell movements of the experimental oysters were continuously recorded by kymographs.

The temperature range covered by these experiments extended from about 0.0° to 38.0° C. The temperatures were usually maintained within ± 0.5° C., and the temperature intervals were 2.0° C. apart. The salinity of the water was usually about 27.0 p.p.t. and the pH, about 7.7.

In all these experiments the oysters were kept in running water, a condition which assured a more normal behavior than if the oysters had been confined to a small container repumping, time after time, the same water which, eventually, could become heavily laden with excretory products. The periods of observation extended from five to seven hours. Therefore, the conclusions offered here refer to these comparatively short periods, and the rates of pumping, as found in these experiments, would not necessarily be representative of longer exposures.

Results

Our studies, an abstract of which has already been offered (Loosanoff, 1950), consisted of 478 individual observations. However, because many oysters either did not open at all or opened but did not pump water, the conclusions are based, actually, on 337 experimental records (Table I).

The lack of activity, as could be expected, was virtually confined to the lower temperatures. Below 2.0° C. only one of eight oysters pumped. It opened its shells when the temperature was only 1.2° C. and, regardless of the somewhat ir-

Table I

Average rates of water pumping, in cc. per hour, of groups of oysters subjected for approximately six-hour periods to temperatures ranging from 0.0° to 38.0° C.

Temperature intervals °C.	Number of oysters			Pumping rate	
	Total	Open	Pumping	Average	Maximum
0.0- 2.0	8	2	1	113	—
2.1- 4.0	52	22	1	863	1,020
4.1- 6.0	28	14	5	180	266
6.1- 8.0	20	12	6	495	1,197
8.1-10.0	36	21	16	763	1,594
10.1-12.0	36	24	22	2,914	4,303
12.1-14.0	32	25	22	3,902	5,409
14.1-16.0	28	27	26	4,344	5,787
16.1-18.0	21	21	21	9,083	11,583
18.1-20.0	33	33	33	7,020	9,773
20.1-22.0	42	42	42	9,802	13,341
22.1-24.0	24	24	24	7,795	10,802
24.1-26.0	14	14	14	9,537	12,635
26.1-28.0	20	20	20	9,366	11,569
28.1-30.0	24	24	24	12,983	15,155
30.1-32.0	19	19	19	11,813	16,253
32.1-34.0	20	20	20	8,948	12,856
34.1-36.0	17	17	17	2,785	4,201
36.1-38.0	4	4	4	2,449	3,884
Total	478	385	337	—	—

regular and feeble shell movements, it produced a weak but, nevertheless, real flow of water. The ability to pump water at low temperature was also recorded for another individual that pumped a relatively large quantity of water at a temperature between 2.7° and 3.3° C. and maintained such a pumping rate for several hours (Fig. 1). Hopkins (1933) working on the closely related species, *Crassostrea gigas*, also reports that the distinct flow produced by this mollusk was observed and recorded at as low a temperature as 2.6° C.

Since some of the oysters exposed to very low temperatures not only kept their shells open, but also pumped small quantities of water, experiments were devised to ascertain whether these oysters were ingesting food under such conditions. They were conducted during February when the temperature of Milford Harbor water is at its lowest. Three-year-old oysters, brought from outdoors, were placed into in-

dividual glass dishes which, in turn, were placed in shallow, white enamel trays of cold, running sea water. A cold culture of deep-green *Chlorella* was added as a biological indicator, to appear in the feces if the oysters fed.

Of the 90 oysters kept for 24 hours at a temperature ranging between 2.0° and 3.0° C., only one individual passed a small quantity of true feces. However, about 15 per cent of the oysters formed pseudo feces. When the temperature was kept between 3.0° and 4.0° C., approximately one-half of the 90 oysters formed pseudo feces, but only a single case of true feces was observed. Thirty oysters from this group were opened and examination showed that in all but one case their stomachs were empty and crystalline styles absent. The same results were obtained when the temperature ranged between 4.0° and 5.0° C. However, when the temperature was kept between 5.0° and 6.0° C., 11 of the 90 oysters expelled true feces, two of them in large quantities, and over 75 per cent formed pseudo feces.

These experiments have shown that, although feeding of oysters below 5.0° C. occurs only as an exception, a relatively high percentage may form pseudo feces,

FIGURE 1. Kymograph record of shell movements (1st line) and rate of pumping (2nd line) of an oyster exposed to water temperature ranging between 2.7° and 4.2° C. from 11:45 A.M. to 6:15 P.M. Each vertical mark of the second line designates the discharge of 235 cc. of water pumped by the oyster.

even at a temperature as low as 2.0° or 3.0° C. The explanation as to why pseudo feces can be produced at lower temperatures than true feces lies, perhaps, in the observation made by Galtsoff (1928a), that the frontal cilia are able to transport the particles along the surface of the gills at a temperature of only about 3.0° C., while the lateral cilia can, as a rule, produce a current only when the temperature of the surrounding water is about 5.0° C.

The observations that some oysters feed at low temperatures are supported by those made by Nomejko and Chanley of Milford Laboratory who, in conducting a survey in Long Island Sound on March 11, 1954, dredged an oyster which possessed a well-developed crystalline style and contained food in its stomach. The temperature of the water at that time was approximately 3.0° C.

Our studies also showed that many oysters which open at the lower temperature may move their shells steadily, but pump no water. Twenty-one of the 22 oysters which opened at temperatures between 2.1° C. and 4.0° C. behaved in this manner (Table I).

Our observations lead us to believe that Galtsoff's (1928a) statement, that in

C. virginica no current is produced and no feeding occurs at or below 5.0° C., should be qualified because this rule, obviously, does not apply to all individuals. His generalizations, nevertheless, remain applicable to the large majority of the oysters.

The average and maximum rates of pumping of the groups of oysters within each temperature interval are given in Table I. In general, the rate of pumping remained low until the temperature interval of 8.0°–10.0° C. was reached. After that, and until the temperature of about 16.0° C. was attained, it showed a moderate increase. No radical fluctuations were recorded, however, between this point and

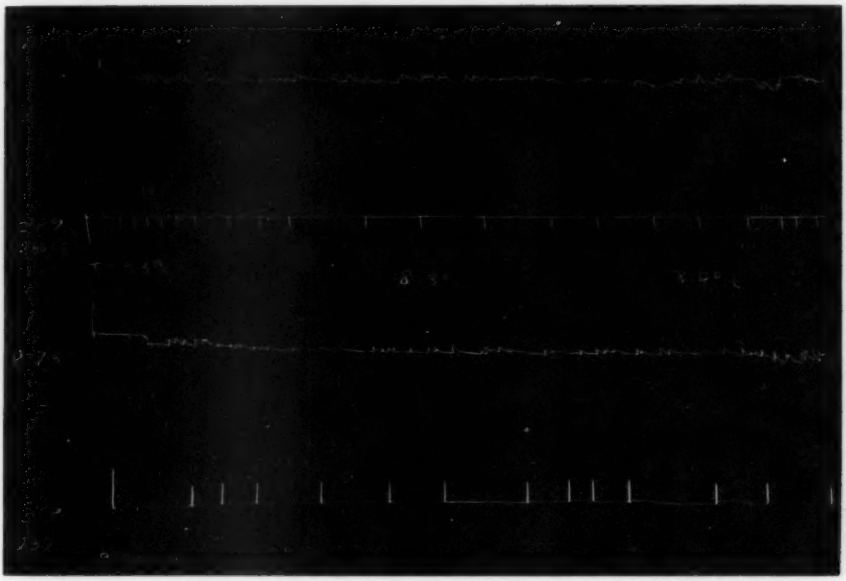

FIGURE 2. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters exposed to temperatures ranging from 8.3° to 9.3° C. from 9:30 A.M. to 2:15 P.M. Each vertical mark on the second line designates the discharge of 280 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 237 cc.

the temperature of 28.0° C. The greatest average rate of pumping, 12,983 cc. per hour, was recorded between 30.0° and 32.0° C. After passing the maximum, the relatively fast pumping continued until about 34.0° C. and then abruptly decreased.

Some aspects of pumping at different temperatures are demonstrated in this article by photographs of the kymograph records showing both shell movements and rates of pumping. At comparatively low temperatures, such as 8.0° to 9.5° C., shell movements of the oysters may not be well defined and the amount of water pumped through the gills remains relatively small (Fig. 2). As the temperature of the water increases toward the optimum range, many oysters begin to display the type of shell movement called "staircase" or "treppe," which some students think

is due to chemical stimulation affecting the experimental oysters with a gradual increase in intensity. We hesitate to accept this as a satisfactory explanation of the phenomenon. In many of our experiments the "staircase" shell movement occurred in normal-feeding oysters, although, as shown in Figure 3, this type of shell movement was often observed soon after the oysters opened and began to pump.

In our studies usually two or four oysters were under simultaneous observation, receiving the same amount of water, connected to the same kymograph and treated identically in all other respects. Yet, in many instances, including the one shown in Figure 4, the "staircase" type of shell movement was displayed at any given time

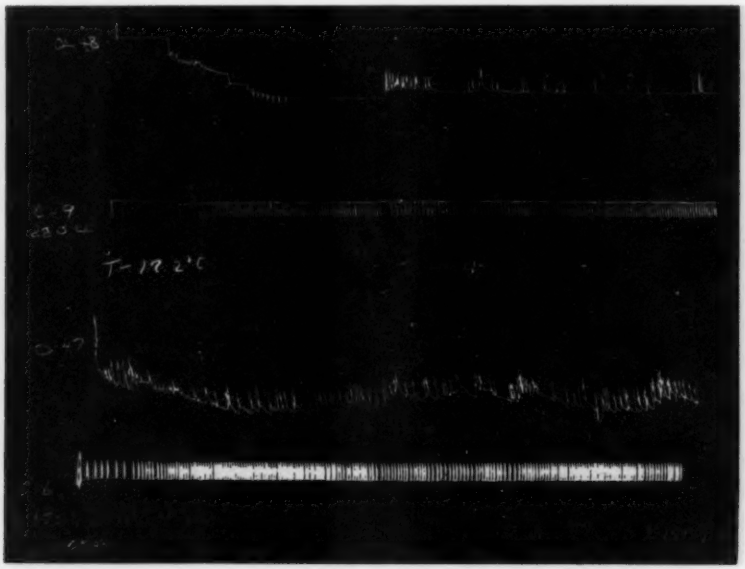

FIGURE 3. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters exposed to temperatures ranging from 17.2° to 17.4° C. from 11:05 A.M. to 4:15 P.M. Each vertical mark on the second line designates the discharge of 280 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 237 cc.

by one oyster only. This clearly indicated that it was not a specific chemical in the water that was responsible for stimulation of the oysters leading to this type of shell movement. Obviously, further physiological studies are needed to explain the cause of the "staircase" type of shell movement.

Within the favorable temperature range many oysters were recorded as pumping over 20,000 cc. per hour, and several individuals were observed pumping at the rate of 25,000 to 29,000 cc. per hour. The maximum rate of pumping for an individual oyster was recorded at a temperature ranging between 24.1° and 24.5° C. when one of the two oysters (indicated as O-10 in Figure 4) averaged 37,446 cc. per hour for a period of about five hours, and for several shorter periods of about 15 minutes

pumped at the rate of about 40,000 cc. per hour. Because of such a rapid rate of pumping the strokes of the needle on the kymograph drum, each representing a discharge of 237 cc. of water, were made so close to each other that the record appeared blurred. Fortunately, by employing the low power of a dissecting microscope the strokes could be accurately counted and the record properly analyzed and evaluated.

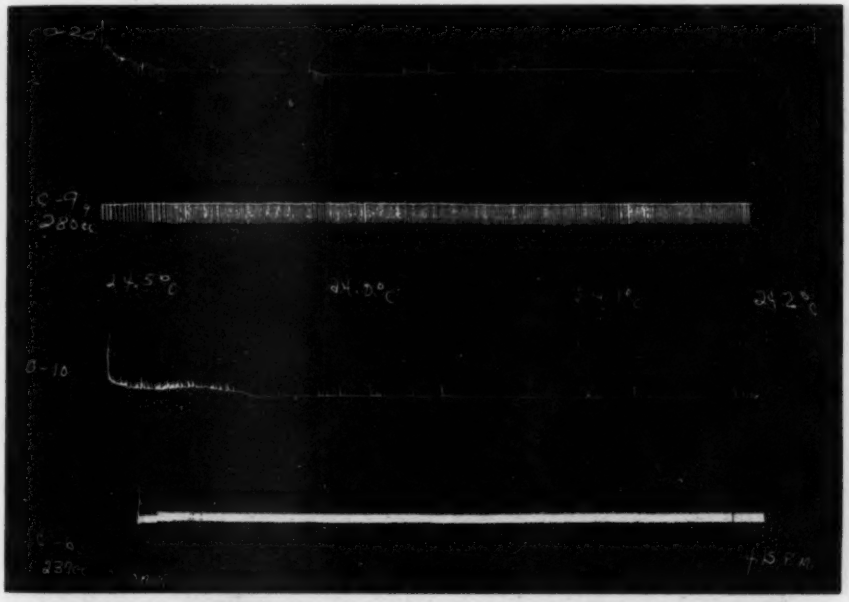

FIGURE 4. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters exposed to temperatures ranging from 24.0° to 24.5° C. from 10:10 A.M. to 4:15 P.M. Each vertical mark on the second line designates the discharge of 280 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 237 cc.

Incidentally, studies of many of our records indicate that often, at favorable temperatures, when the shells of the oysters are open and relatively motionless, large quantities of water are pumped. This is well demonstrated by the activities of the two oysters in Figure 4, one of which pumped the record quantities of water and the other also pumped at a rapid rate.

Almost immediately after passing the temperature level of 32.0° C. and, actually, in some instances, even between the temperatures of 31.1° and 32.0° C., certain signs began to appear, indicating that some oysters were being unfavorably affected. Among these signs were changes in the character of the shell movements, complete closing of the shells and cessation of pumping at frequent intervals, and a reduced rate of pumping even during the period when the shells remained open. Within the range of 34.1° to 36.0° C. these symptoms became more pronounced (Fig. 5).

Above 36.1° C. the type of shell movement became even more abnormal, and the oysters stayed closed approximately two-thirds of the time exposed.

To determine the significance of the differences in the average pumping rates of oysters at the different temperature intervals, a simple analysis of variance was employed. It was found unnecessary to compute all the *t*'s between adjacent temperature intervals. The same objective could be achieved by using a simple analysis of variance to test for homogeneity within each of the several apparently homogeneous levels, and also to test for the significance of the differences between these levels. These tests showed homogeneity within the following five temperature intervals: 4.1°–10.0° C., 10.1°–16.0° C., 16.1°–28.0° C., 28.1°–34.0° C., and 34.1°–38.0° C. The means for these five levels are shown in Figure 6 and are 593; 3,714; 8,727;

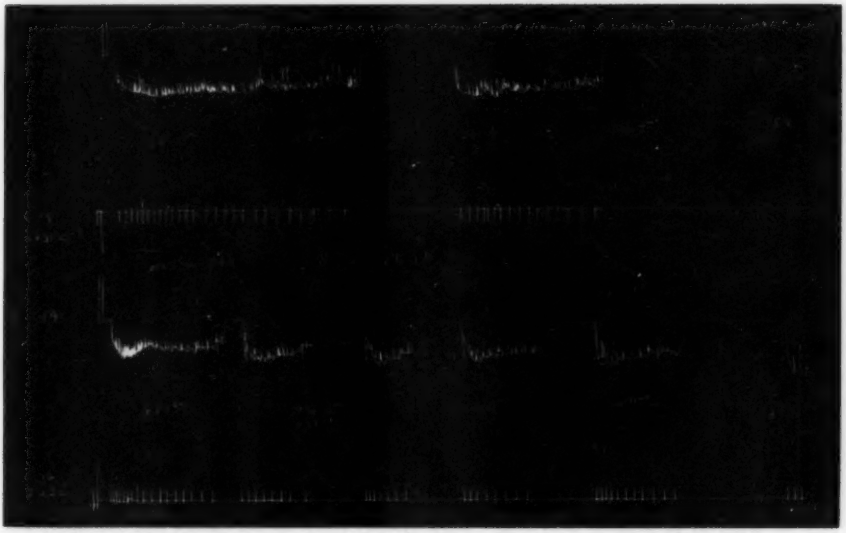

FIGURE 5. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters exposed to temperatures ranging from 34.9° to 35.2° C. from 11:15 A.M. to 6:40 P.M. Each vertical mark on the second line designates the discharge of 268 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 232 cc.

11,365 and 2,762 cc., respectively. The analysis indicated, as expected from inspection of this figure, that there were highly significant differences among these five levels. It was concluded, therefore, that the average pumping rate can be described as being related to these differences.

There are ecological situations in nature where changes in the temperature of the water are frequent and rapid. Many small, shallow, salt water ponds, of the type that can be adapted to shellfish cultivation, may belong to this category. Oysters living in such ponds or other similar bodies of water may experience such changes in temperature, especially during early spring and late autumn when the temperature of the water may quickly cool off during the night, decreasing to almost

0.0° C., and rapidly rise during the daytime. The question that naturally arises for such a situation is, how are the oysters affected by such changes and do they resume normal pumping regardless of the rapid change in the temperature from nearly freezing to about 15.0° C. or even higher?

The first series of experiments designed to answer the above question was conducted in October. The oysters were brought from outdoor tide-filled tanks, where the temperature of the water was about 13.0° C. One-half of the oysters, which served as the control, were placed in an aquarium in which the water temperature

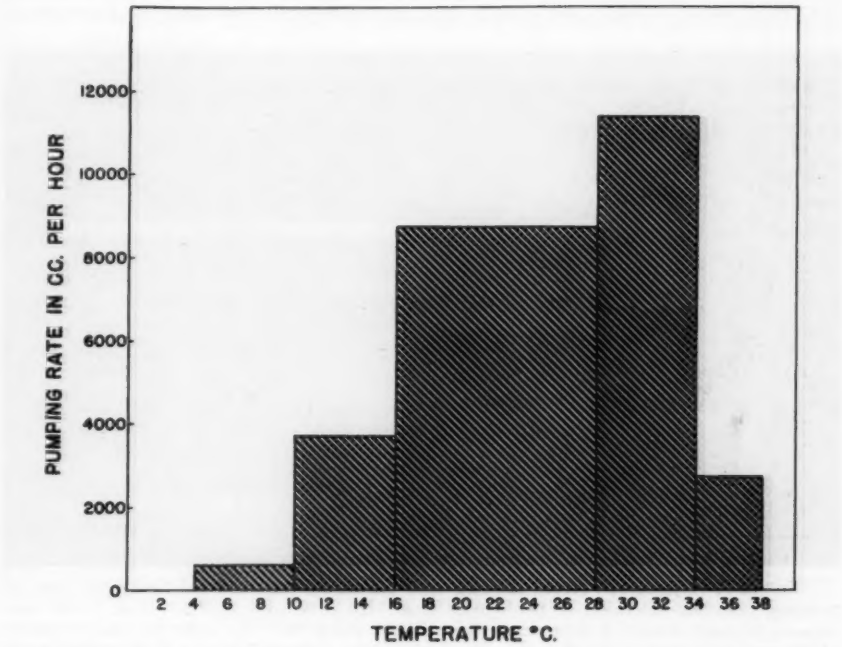

FIGURE 6. Mean rate of water pumping by oysters at five homogeneous temperature levels.

was kept at about 18.0° C. The other half were put in special containers in the refrigerator, where the temperature was about 3.0° C., and kept there from eight to 12 days. After this period at low temperature these oysters, as well as those serving as the control, were attached to kymographs. Extreme care was exercised during the handling to maintain the low temperature of the water for the refrigerated oysters. When the oysters of both groups were ready and attached to the kymographs a flow of water, about 18.0° to 20.0° C., was introduced simultaneously to all chambers, displacing the cold water surrounding the refrigerated oysters, and the behavior of all specimens was recorded from then on.

In a second series of experiments conducted in the middle of the winter (Fig. 7) the low-temperature oysters were not kept in the refrigerator but in the outdoor

tanks where the temperature of the water was near 0.0° C. The oysters which were to serve as the control were, however, kept for three weeks in the laboratory at room temperature before being used in the experiments. In all other respects the methods used in the two series were the same. Altogether, in the two series of experiments, 33 control and 31 experimental oysters were used.

Student's "*t*" test was made to determine the significance of differences in the average pumping rate of the two groups. Because the "*t*'s" were far from significant, it was concluded that sudden upward changes in temperature, such as from 1.5° C. to about 18.0° C. (Fig. 7), had no significant effect on the pumping rate of the "cold water" oysters when such rate was compared with that of the control

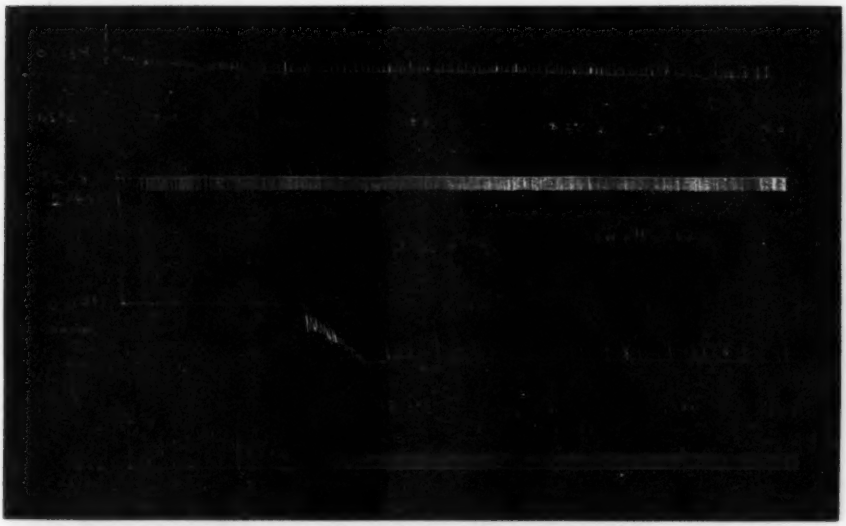

FIGURE 7. Kymograph record showing shell movements (1st and 3rd lines) and rates of pumping (2nd and 4th lines) of two oysters from 9:25 A.M. to 4:30 P.M. One, O-134, was exposed to rapid change of temperature from 1.5° to about 18.0° C., while the control oyster, O-141, was constantly subjected to the same temperature of about 18.0° C. Each vertical mark on the second line designates the discharge of 236 cc. of water pumped by the oysters, while each mark of the fourth line shows the discharge of 251 cc.

oysters. It was demonstrated, nevertheless, that there was a highly significant tendency of the oysters subjected to sudden changes from low to high temperature to open and pump almost immediately after the change was made, while the control oysters, as illustrated in Figure 7, usually required a much longer time to open their shells and begin pumping.

The results of these experiments differ from those of Galtsoff (1946) who, after keeping two oysters for 24 hours at 5.0° C. and then exposing them to a temperature of about 21.0° or 22.0° C., concluded that the shell movements of those oysters were abnormal and that they started to pump only on the third or fourth day after they had been placed in warmer water. We found no such abnormalities in the

shell movements of the individuals used in our experiments (Fig. 7). Moreover, as already mentioned, the majority of our oysters previously kept in cold water opened and began pumping almost immediately, not after three or four days as found by Galtsoff. Our observations indicated, consequently, that the response of the oysters to a sharp and quick increase in temperature, from near 0.0° to about 15.0° to 20.0° C., is rapid and results in apparently normal pumping. The oysters, therefore, are physiologically well adapted to such changes which they, no doubt, often encounter in nature, for example, when living on the tidal flats where, at the autumn low tide, the night air may cool the water of the small pools containing the oysters almost to freezing, while the incoming tide may cover the same oysters with much warmer water.

Discussion

The rate of water pumping of different bivalves has been studied by a number of investigators. Among them, Jørgensen (1949) and Willemsen (1952) studied it in the common mussel, *Mytilus edulis;* Fox *et al.* (1937) and Rao (1953) worked on the closely related species, *M. californianus;* Willemsen (1952) also determined the quantities of water pumped by cockles, *Cardium edule;* Hopkins (1933, 1935) conducted extensive studies on the pumping of the Japanese commercial oyster, *Crassostrea gigas,* while Chipman and Hopkins (1954) determined the rate of water filtration by the common scallop, *Pecten irradians.* Most of these workers, except Hopkins, used the so-called indirect method based on a reduction of the number of particles or plankton organisms in suspension in the water in which the mollusks were kept. Hopkins used his own "cone" method by means of which he could determine only the relative rate at which the oysters pumped at different temperatures.

The most extensive observations on the rate of water transport were conducted on the American oyster, *C. virginica.* Except for Galtsoff's (1928a) pioneer experiments and Jørgensen's (1952) studies, investigators have used the direct or so-called "rubber apron" method, which permits measuring the actual quantities of water passed by oysters without interfering with their normal behavior. Most of these studies, however, including those of Loosanoff and Nomejko (1946), Loosanoff and Engle (1947) and Galtsoff *et al.* (1947), were made within relatively narrow temperature ranges not including the low or high temperature zones. Results of studies by Nelson (1935) and Loosanoff (1950), covering broader ranges, were presented only as brief abstracts.

As already mentioned, Galtsoff's (1928a) early experiments were criticized because the methods that he was then employing adversely affected the oysters (Nelson, 1938). Yet, regardless of the defects in his methods, Galtsoff, although giving rather low figures for the quantities of water pumped by oysters, indicated, nevertheless, quite correctly the general trend of the changes in the rate of pumping in relation to different temperature levels. For example, he observed that no feeding took place at 5.0° C. or lower. These conclusions, with the few exceptions demonstrated by our studies and by Hopkins' (1933) observations on the closely related species, still hold true for the majority of the population of *C. virginica* of northern waters. Galtsoff's observations that between 15.0° and 25.0° C. the fluctuations in the rate of pumping of individual oysters were small, strongly support the validity of our conclusions that between 16.0° and 28.0° C. the rate of pumping showed no marked fluctuation. Finally, his figures indicate a sharp drop in the rate of pump-

ing after the temperature reaches and passes 34.0° C., as was also observed in our experiments.

Our observations showed that the highest average rate of flow of water through the gills of the oysters was achieved at about 29.0° C. (Table I). In this respect our results are in agreement with those of Nelson (1935) who determined that the maximum pumping in oysters occurs near 30.0° C. Our conclusions also resemble those of Galtsoff (1928b) that the optimum temperature for the mechanical activities of the oyster gills lies between 25.0° and 30.0° C. However, Galtsoff found that the maximum rate of pumping occurs at 25.0° C., somewhat lower than that recorded by Nelson (1935) or found in our studies. Nevertheless, considering Galtsoff's findings concerning the temperature at which the maximum activity of the oyster gills takes place, the three series of studies are in close agreement. Collier (1954) thinks, however, that even on the Gulf Coast the optimum temperature range for *C. virginica* lies between 15.0° and 25.0° C.

As to the maximum quantities of water pumped by *C. virginica,* there is considerable disagreement between the conclusions of the earlier workers, whose studies were reviewed by Galtsoff (1928a), and the more recent ones. The former estimated that oysters are capable of pumping only a few liters per hour. Wells (1926) gave the highest figure, advancing the opinion that at a favorable temperature oysters can average more than 7.5 liters per hour. His conclusions, however, were strongly challenged by Galtsoff (1928a), who expressed doubt that oysters can pump water at that rate.

Later investigators, who used the "rubber apron" method, have shown that the quantities of water pumped by oysters are much greater than those suggested by earlier workers. Nelson (1935) gives 26.0 liters per hour as the maximum quantity. Our maximum figure for a single oyster, as already mentioned, was 37,446 cc. for one complete hour, and an even higher rate was recorded for shorter periods. The maximum average hourly rate of pumping for a group of oysters was recorded within the temperature range of 28.1° to 30.0° C. and was 12,983 cc.; the maximum hourly rate, recorded between 30.1° and 32.0° C., was 16,253 cc. (Table I). Galtsoff *et al.* (1947) gave 9.6 liters as the average and 16.0 liters as the maximum hourly rate of water pumped by the oysters of York River, Va. Jørgensen's (1952) figures obtained by somewhat different methods are, nevertheless, very close to ours, being 11.0 and 15.5 liters per hour for the average and maximum rates, respectively. These figures attest the ability of the oysters to pump large quantities of water and indicate the importance of considering the water requirements of these mollusks in experimenting with them, or in their cultivation.

Summary

1. Some adult Long Island Sound oysters are able to pump water at a temperature as low as about 1.0° C. Oysters with crystalline style and food in their stomachs may occasionally be found in northern waters in winter.

2. Approximately 15 per cent of the oysters exposed to temperatures ranging from 2.0° to 3.0° C., and approximately 50 per cent of the oysters kept between 3.0° and 4.0° C. formed pseudo feces.

3. The average and maximum rates of pumping of the groups of oysters exposed to temperatures from 0.0° to 38.0° C. were determined. The rate remained low

under 8.0° C. Within the range from 8.1° to 16.0° C. the rate steadily increased. Between that point and about 28.0° C. the rate showed no marked fluctuation. A further increase was noted between 28.1° and 32.0° C. It is within this range that the maximum average rate of pumping of 12,983 cc. per hour was recorded. Between 32.1° and 34.0° C. the rate was also rapid. Beyond 34.1° C. the oysters showed a marked decrease in the rate of pumping and their shell movements were abnormal.

4. The maximum rate of pumping for an individual oyster averaging 37,446 cc. per hour was recorded at the temperature of about 24.0° C. For short periods of five to 15 minutes the rate of pumping of the same oyster exceeded 40,000 cc. per hour.

5. Statistical tests showed homogeneity of the rates of pumping within the following five temperature intervals: 4.1°–10.0° C., 10.1°–16.0° C., 16.1°–28.0° C., 28.1°–34.0° C. and 34.1°–38.0° C. The means for these five intervals were 593; 3,714; 8,727; 11,365 and 2,762 cc. per hour, respectively. Highly significant differences among these five levels were indicated by statistical analysis.

6. The rate of pumping of oysters kept at a temperature below 5.0° C. and then quickly changed to the higher temperature of 18.0° to 20.0° C. was virtually the same as the control oysters, thus indicating that the response of the oysters to such changes in environment and their adjustment to these changes are rapid.

LITERATURE CITED

CHIPMAN, W. A., AND J. G. HOPKINS, 1954. Water filtration by the bay scallop, *Pecten irradians*, as observed with the use of radioactive plankton. *Biol. Bull.*, **107**: 80–91.

COLLIER, A., 1954. A study of the response of oysters to temperature, and some long range ecological interpretations. *Nat. Shellfish. Assoc. Conv. Address*, 1953: 13–38.

FOX, D. L., H. N. SVERDRUP AND J. P. CUNNINGHAM, 1937. The rate of water propulsion by the California mussel. *Biol. Bull.*, **72**: 417–438.

GALTSOFF, P. S., 1926. New methods to measure the rate of flow produced by the gills of oyster and other molluscs. *Science*, **63**: 233–234.

GALTSOFF, P. S., 1928a. Experimental study of the function of the oyster gills and its bearing on the problems of oyster culture and sanitary control of the oyster industry. *Bull. Bur. Fish.*, **44**: 1–39.

GALTSOFF, P. S., 1928b. The effect of temperature on the mechanical activity of the gills of the oyster (*Ostrea virginica* Gm.). *J. Gen. Physiol.*, **11**: 415–431.

GALTSOFF, P. S., 1946. Reaction of oysters to chlorination. *U. S. Fish and Wildlife Service, Research Report*, **11**: 1–28.

GALTSOFF, P. S., W. A. CHIPMAN, JR., J. B. ENGLE AND H. N. CALDERWOOD, 1947. Ecological and physiological studies of the effect of sulphate pulp mill wastes on oysters in the York River, Virginia. *Fish. Bull. 43, U. S. Fish and Wildlife Serv.*, **51**: 57–186.

HOPKINS, A. E., 1933. Experiments on the feeding behavior of the oyster, *Ostrea gigas*. *J. Exp. Zool.*, **64**: 469–494.

HOPKINS, A. E., 1935. Temperature optima in the feeding mechanism of the oyster, *Ostrea gigas*. *J. Exp. Zool.*, **71**: 195–208.

JØRGENSEN, C. B., 1949. The rate of feeding by *Mytilus* in different kinds of suspension. *J. Mar. Biol. Ass.*, **28**: 333–344.

JØRGENSEN, C. B., 1952. On the relation between water transport and food requirements in some marine filter-feeding invertebrates. *Biol. Bull.*, **103**: 356–363.

JØRGENSEN, C. B., 1955. Quantitative aspects of filter-feeding in invertebrates. *Biol. Rev.*, **30**: 391–454.

LOOSANOFF, V. L., 1950. Rate of water pumping and shell movements of oysters in relation to temperature. *Anat. Rec.*, **108**: 132.

LOOSANOFF, V. L., 1954. New advances in the study of bivalve larvae. *Amer. Sci.*, **42**: 607–624.

LOOSANOFF, V. L., AND H. C. DAVIS, 1950. Conditioning *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. *Biol. Bull.*, **98**: 60–65.

LOOSANOFF, V. L., AND J. B. ENGLE, 1947. Effect of different concentrations of micro-organisms on the feeding of oysters (*O. virginica*). *Fish. Bull. 42, U. S. Fish and Wildlife Serv.*, **51**: 31–57.

LOOSANOFF, V. L., AND C. A. NOMEJKO, 1946. Feeding of oysters in relation to tidal stages and to periods of light and darkness. *Biol. Bull.*, **90**: 244–264.

MOORE, H. F., 1910. Volumetric studies of the food and feeding of oysters. *Bull. U. S. Bur. Fish.*, **28**: 1295–1308.

NELSON, T. C., 1935. Water filtration by the oyster and a new hormone effect thereon. *Anat. Rec.*, **64**: 68.

NELSON, T. C., 1936. Water filtration by the oyster and a new hormone effect upon the rate of flow. *Proc. Soc. Exp. Biol.*, **34**: 189–190.

NELSON, T. C., 1938. The feeding mechanism of the oyster. I. *J. Morph.*, **63**: 1–61.

RAO, K. P., 1953. Rate of water propulsion in *Mytilus californianus* as a function of latitude. *Biol. Bull.*, **104**: 171–181.

VERWEY, J., 1952. On the ecology of distribution of cockle and mussel in the Dutch Waddensea, their role in sedimentation and the source of their food supply. *Arch. Néerl. de Zool.*, **10**: 172–239.

WELLS, W. F., 1926. Conclusions reached by the U. S. Public Health Service in experiments at Fisherman's Island. *Amer. J. of Pub. Health*, **16**: 10–12.

WILLEMSEN, J., 1952. Quantities of water pumped by mussels (*Mytilus edulis*) and cockles (*Cardium edule*). *Arch. Néerl. de Zool.*, **10**: 153–160.

QUANTITATIVE ASPECTS OF DEOXYRIBOSE NUCLEIC ACID (DNA) METABOLISM IN AN AMICRONUCLEATE STRAIN OF TETRAHYMENA [1]

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Of extreme cytological and genetic interest is the heteronucleate condition—the presence of both micro- and macronuclei—in most ciliate protozoans. The micronucleus is a permanent cell organelle, normally diploid, which divides mitotically during vegetative cell growth, and meiotically during conjugation or autogamy. On the other hand, the macronucleus, which contains far more chromatin as indicated by its size and staining capacity, appears to pull apart amitotically during vegetative growth; during conjugation or autogamy, it degenerates. Following this phenomenon, the new macronucleus develops from a division product of the synkaryon, presumably as a result of endomitosis (as demonstrated by Grell, 1953a, in a suctorean, *Ephelota gemmipara*).

Such obvious differences between these two types of nuclei led to the earlier belief that they contained two types of chromatin—idiochromatin in the micronucleus, and trophochromatin in the macronucleus (as noted by Wichterman, 1953). By a cytochemical study of vegetative individuals of *Paramecium caudatum*, however, Moses (1950) found that, although the macronucleus contains perhaps 40 times as much nucleoprotein as the micronucleus, both types of nuclei contain very similar *relative* amounts of deoxyribose nucleic acid (DNA), ribose nucleic acid and protein, which would seem to indicate that both are metabolically active. More recently, however, a difference in protein composition has been reported by Alfert and Goldstein (1955) for another ciliate, *Tetrahymena pyriformis* (mating types I and II, Elliott). Using a recently developed, direct staining method for basic protein (Alfert and Geschwind, 1953), they found the ratio of basic protein: DNA in the micronucleus to be about 1.7 times greater than that in the macronucleus.

As a result of its large amount of chromatin, the macronucleus might be expected to have more genetic influence than the micronucleus. This has, indeed, been found by Sonneborn (1947) to be the case in *Paramecium aurelia*. Organisms with a micronucleus of one genotype and a macronucleus of another (resulting from regeneration, after conjugation, of a part of the old macronucleus) show the characteristics carried by the macronucleus. He has referred to the micronucleus as the germinal nucleus, and the macronucleus as the somatic nucleus. More recently, Sonneborn (1954) has clearly demonstrated that genes in the micro-

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nucleus may be completely inactive. Phenotypic expression following conjugation in ciliates is, in fact, most complex. In *Paramecium* and *Tetrahymena*, organisms of identical genotype may differ as to phenotype (for example, mating type—Sonneborn, 1939; Nanney and Caughey, 1953). In some cases, differentiation appears to be influenced by the cytoplasm, which of course had been influenced by the pre-conjugation macronucleus (Sonneborn, 1951, 1953; Nanney 1953a, 1956, 1957). It appears that location in the cytoplasm determines which division products of a synkaryon will be micro- and which will be macronuclei, in both *Paramecium* (Sonneborn, 1953) and *Tetrahymena* (Nanney, 1953b).

As exceptions to the rule that ciliates normally contain both types of nuclei, races and individuals occur in which micronuclei are absent. Such amicronucleate organisms, of course, cannot undergo autogamy, although they may conjugate with normal organisms (Chen, 1940; Sonneborn, 1953), and receive from them half a set of micronuclear chromosomes. Some amicronucleate strains are capable of vegetative growth for extended periods of time. Experimental strains of *Tetrahymena pyriformis*, for the most part, have no micronuclei (Corliss, 1953). In contrast, amacronucleate strains of ciliates have not been reported. Seshachar and Dass (1953) have observed in a peritrich, *Epistylis articulata*, that some daughter cells, receiving no macronucleus but only a micronucleus as a result of abnormal fission, eventually regenerated a macronucleus from a division product of the micronucleus. These various circumstances indicate that whereas the macronucleus is essential to the organism, the micronucleus is not. In amicronucleate strains, the macronucleus rather than the micronucleus is necessarily a permanent cell organelle.

In the present experiments, quantitative aspects of DNA metabolism in the macronucleus have been investigated in an amicronucleate strain of *Tetrahymena pyriformis*—strain H, isolated by Hetherington in 1930 (1933). Having no micronucleus, this strain has not been observed to undergo conjugation or autogamy, with the attendant degeneration and subsequent reformation of the macronucleus.

By means of microspectrophotometric analysis, the amount of DNA (indicated by the uptake of Feulgen dye, which has been shown to be proportional to the total DNA—Swift, 1953) has been determined at different stages of growth. The answers to a number of questions have been sought. For example, in the apparent absence of a mitotic apparatus, does the macronucleus divide equally between daughter cells? Before the next division, is the DNA precisely duplicated, as in mitotically dividing cells (Swift, 1950), or does an indefinite amount of synthesis occur? Does the DNA remain stationary in cells of a mass culture which has reached maximum growth? Finally, during what part of the growth cycle does the macronucleus synthesize DNA?

Methods

I. *Stock cultures*

The stock of *Tetrahymena pyriformis* H used in these experiments was obtained from Mr. Sheldon Greer of the Department of Zoology, Columbia University, who in turn had obtained it from Dr. Seymour Hutner of the Haskins Laboratories, New York. The cells were cultured in 5-ml. amounts of 2% proteose peptone broth (Difco) in 16 × 150 mm. Pyrex test tubes. The tubes were arranged in a slanting position in a 25° C. constant temperature room. Stock cultures were

transferred weekly (0.1-ml. inocula), at which time samples were spread on proteose peptone agar to test for possible contamination.

Cultures for experimental purposes were inoculated on the day before they were to be used, to insure that the cells would be in the logarithmic stage of growth.

II. *Fixation and staining of mass cultures*

Cells from a culture growing in a test tube were fixed directly on a chemically clean microscope slide, which was first placed in a paraffin-coated paper box (80 mm. long, 26 mm. wide, 12 mm. deep). After 10 ml. fixative (9 parts absolute alcohol: 1 part glacial acetic acid) were added, 0.25-0.5 ml. of the culture was delivered slowly from a fine-tipped pipette over the slide. In 10 minutes the slide was removed, drained, and held on its side against a paper towel while absolute alcohol was delivered slowly along the other edge from a fine-tipped pipette. The slide was then placed in absolute alcohol. After 10 minutes or longer, the slide was drained, 0.5% celloidin was run over the surface to which the cells were attached, and the slide was again drained and placed in 80% alcohol (modified from Chen, 1944). Throughout the subsequent treatment of the slide, precautions were taken so that the celloidin would not become dry and thus loosened from the slide.

Nuclei were stained according to the Feulgen procedure essentially as described by Di Stefano (1948) except that they were hydrolyzed in 2 N hydrochloric acid at 40° C. instead of in 1 N hydrochloric acid at 60°. This modification was used so that the time of hydrolysis would be less critical, since cells on different slides were to be compared. Photometric measurements indicated 55 minutes to be the optimal hydrolysis time.

After treatment with Feulgen preparation and bleach, the slide was rinsed during 5 minutes in 5 changes of water. The slide was run up through the alcohols to 1 part ether: 1 part absolute alcohol (which removed the celloidin), through absolute alcohol and xylol, and finally was mounted in oil of refractive index 1.572, which closely matched the refractive index of the cells.

III. *Growth of individual cells*

In a set of experiments to determine the generation times in small clones, individual cells were isolated under sterile conditions to hanging drops of proteose peptone broth. Growth was followed by observations every 15 minutes through a dissecting microscope.

Although the hanging drop method was quite satisfactory for following growth, it was not practical if the cells were to be fixed; when the cover slip was removed, the small drop of broth dried up very quickly. A different method was used to grow the cells in larger drops of medium (about 0.02 ml.) in small watch glasses (U. S. Bureau of Plant Industry Model, A. H. Thomas). These dishes (coated with silicone from Dow-Corning Sight Savers before sterilization to prevent the drops from spreading) were kept in the moist chamber of a petri plate lined top and bottom with wet filter paper in which holes had been cut for viewing the drops. To prevent fogging of the inner surface of the petri top, it was treated with a paste (Clersite, Chicago, Ill.) applied with sterile Wipettes.

For convenience, individual cultures were grown in the laboratory, at temperatures varying from 24–27°, averaging 25° C.

IV. *Fixation and staining of individual cells*

Individual containers for the fixation of cells from small clones were prepared by sealing paraffin rings to slides. The rings were formed by dipping a silicone-coated vial (10 mm. outside diameter), filled with cold water, into molten paraffin, and then into cold water. The paraffin coat was cut into rings about 5 mm. deep, which were slipped off the vial and sealed to the slide by careful heating. Two

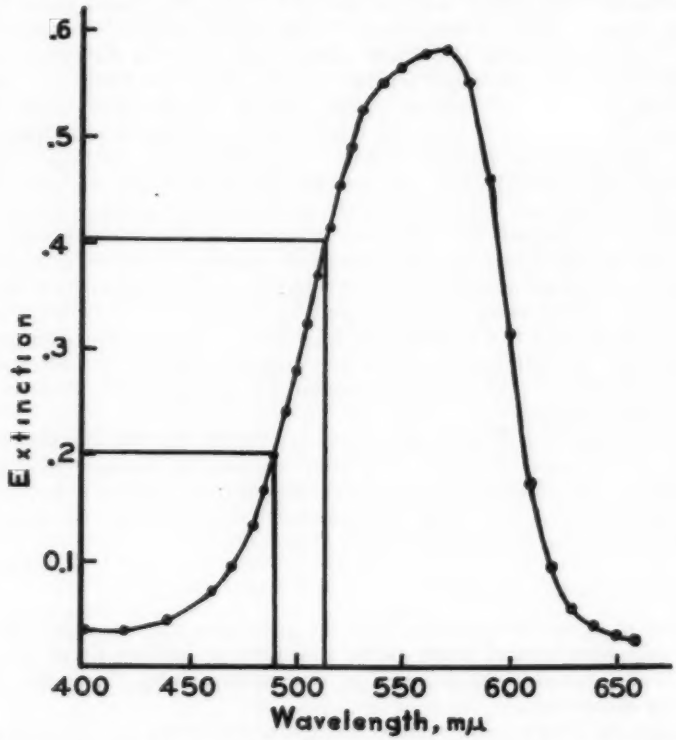

FIGURE 1. Absorption curve of a *Tetrahymena* macronucleus stained with Feulgen dye. The two wave-lengths (490 mμ and 514 mμ) used in making photometric measurements are indicated.

were placed on each slide, directly above notched circles which had been drawn on the opposite side of the slide with a diamond pencil. The notches, facilitating the location of cells, could be seen at the inner edges of the paraffin rings.

Fixation was carried out under two dissecting microscopes—one for the isolation of the cell, and one for its fixation. About 0.4 ml. of fixative was placed in one of the paraffin containers. The cell was delivered carefully from a micropipette at the surface of the slide, and a coverslip was placed on top of the con-

tainer to minimize evaporation which would cause agitation of the fixative. After 10 minutes the fixative was removed with a pipette (whose fine tip was drawn out at an angle), and absolute alcohol was then carefully added. Usually this procedure resulted in sticking the cell to the slide; occasionally the cell became loose, in which case the alcohol was removed, and fresh alcohol was added. In this way the majority of cells could be saved.

After cells had been fixed in both containers on a slide, notations were made of the cells' locations and the slide was placed in a petri plate containing absolute alcohol. Within an hour the paraffin rings became loosened and were removed with a forceps. At this time the presence of the cells was checked under a dissecting microscope. The slide was rinsed in xylol-alcohol to remove any adhering paraffin molecules, in absolute alcohol, and then celloidin was run over its surface

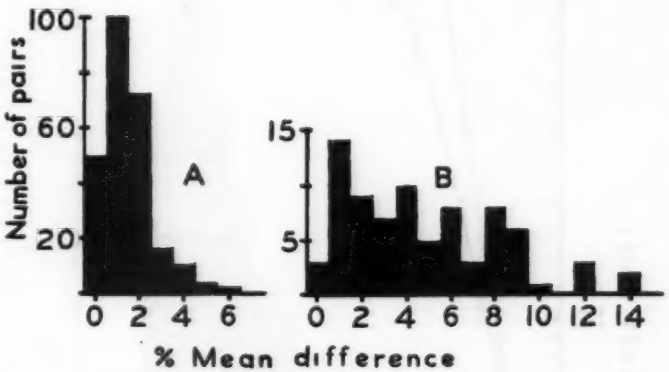

FIGURE 2. Variability of duplicate DNA determinations compared with variability of DNA content in sister cells. (A) % Mean difference in 263 pairs of duplicate DNA determinations. Range 0.0 to 5.7%, mean 1.4%. (B) % Mean difference of DNA values in 83 pairs of sister cells. Range 0.1 to 14.2%, mean 4.7%.

as previously described. Subsequent treatment of the slide differed from that already described only in that the celloidin was removed, after staining, by placing the slide in a petri plate containing ether-alcohol, when the presence of the cells was again checked under the microscope.

V. *Photometric methods*

Measurements were made with the microspectrophotometric apparatus described by Pollister (1952), using a Bausch & Lomb monochromator. Because it was desired to measure all the small clones fixed, and many of the nuclei were not spherical, the two-wave-length method described by Ornstein (1952) and Patau (1952) was used. Figure 1 shows a typical absorption curve for a Feulgen-stained nucleus. The two wave-lengths selected were 490 mμ and 514 mμ (which were also used by Patau). For a single DNA determination, two readings through both nucleus and background were made at each wave-length. Two determina-

tions were made and averaged for each cell from a small clone; between determinations, the fine adjustment and the condenser of the microscope were refocused, and the phototube diaphragm was readjusted. The % mean difference of duplicate determinations on 263 cells ranged from 0 to 5.7, with an average of 1.4 (Fig. 2A).

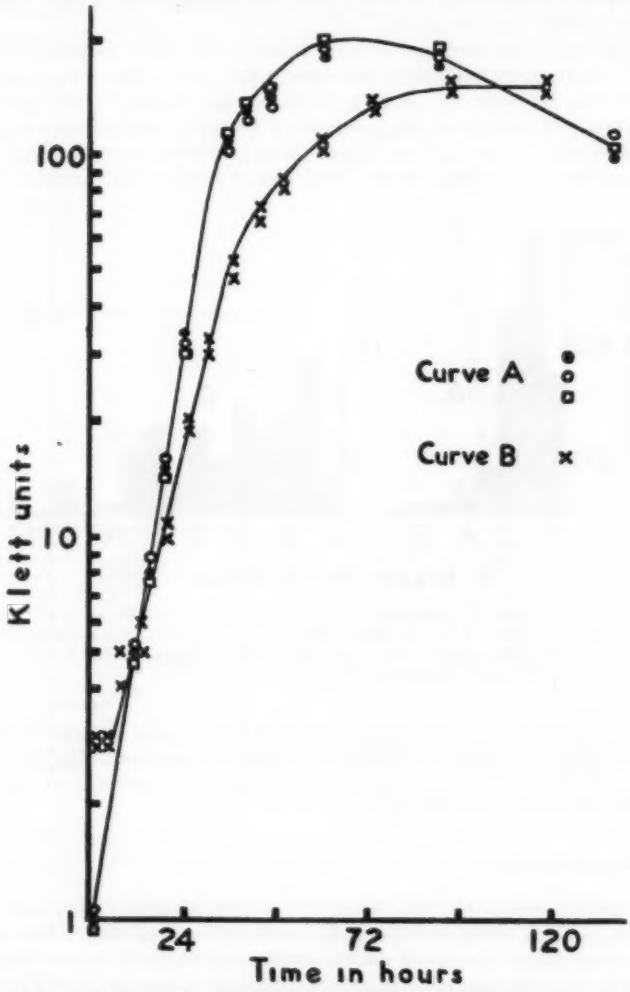

FIGURE 3. Growth curves for *Tetrahymena pyriformis* H. Both curves are drawn from turbidity readings in a Klett photoelectric colorimeter. Curve A is based on three tubes of cells grown in a roller. Curve B is based on a series of tubes, slanted but not otherwise aerated. Maximum growth for both A and B was about 3×10^6 cells per ml. (Although all the growth tubes were the same size, two different sizes of tubes were used for the two sets of readings, accounting for the different heights of the curves.)

I. *Growth of Tetrahymena*

The ability of *Tetrahymena* to thrive under controlled conditions in non-particulate, sterile medium makes it an ideal protozoan for biological research. Although proteose peptone broth was used in this investigation, *Tetrahymena* can be grown also in media of carefully measured, known constituents (Elliott and Hayes, 1953; Kidder, Dewey and Heinrich, 1954). The growth response in both proteose peptone and defined media is quite reproducible.

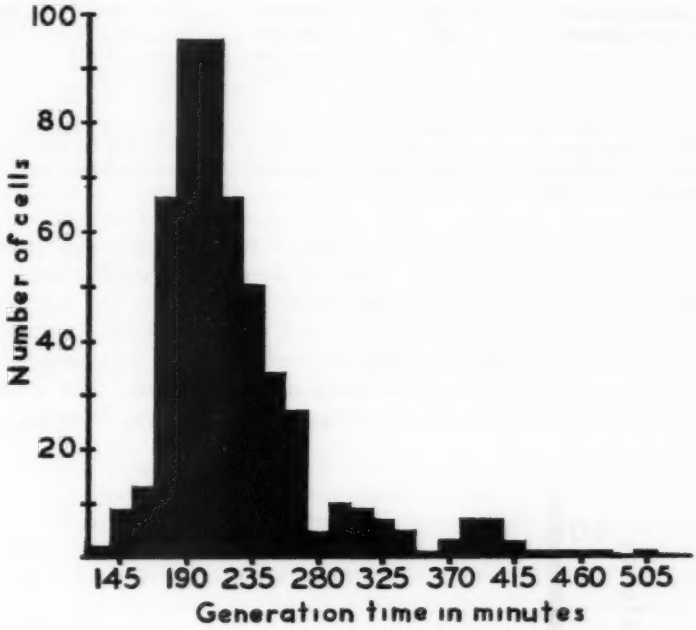

FIGURE 4. Generation times in isolated clones. A total of 515 cells (160 two-cell clones and 195 individual cells) are represented. Data for the different groups of cells included in this histogram are given in Table I.

A. *Growth in mass cultures.* *Tetrahymena* shows a pattern of growth similar to that for other micro-organisms—a short lag period (when the inoculum is from a culture in stationary phase); a logarithmic stage of growth, during which the cell number doubles in regular time intervals; a period of deceleration, when the rate of cell divisions decreases; and a stationary period, during which the number of cells remains constant, with few cell divisions or deaths.

In Figure 3 are shown two different growth curves for cells grown in 2% proteose peptone broth, based on turbidity readings in a Klett photoelectric colorimeter. Curve A represents the growth response of three tubes of cells (inocula

TABLE I

Generation times of individual cells

Growth conditions	Number of cells	Range (min.)	Mean ± S.D. (min.)	Mode (min.)
Hanging drops	170 (includes 84 2-cell clones)	135–505	215 ± 53.7	190–205
Drops in watch glasses	152 (76 2-cell clones)	150–465	220 ± 58.1	190
Drops in watch glasses*	90	155–480	240 ± 60.2	205
Drops in watch glasses*	103	150–400	227 ± 43.7	205
Total	515	135–505	223 ± 53.0	190–205

* These generation times were observed in the course of studying DNA synthesis (see Results, IIB3). Photometric measurements were made of the 90 cells in line 3; the 103 cells in line 4, which received identical treatment, were not measured (because of cell loss, particles overlying a nucleus, or a broken nucleus).

from a log phase culture) aerated in a roller. The generation time during log phase is seen to be about four hours. Curve B shows the response of cells (inocula from stationary phase) grown undisturbed in slanted tubes but not otherwise aerated—the conditions under which stock and mass experimental cultures were grown in the present experiments. The two readings at each time period of Curve B represent two tubes of a series inoculated at the same time; after a tube was shaken to distribute the cells, and a reading was made, it was discarded. Curve

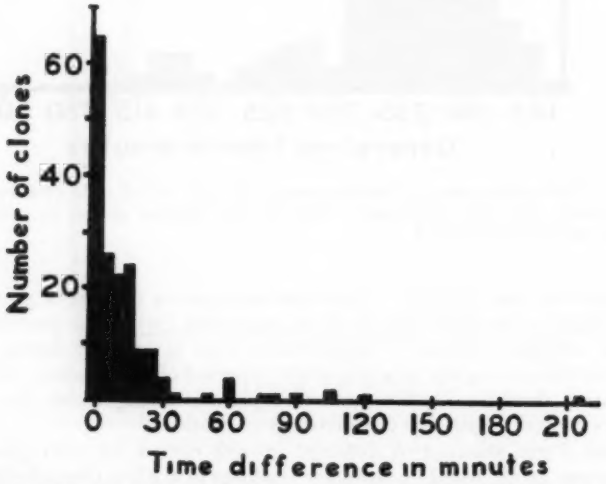

FIGURE 5. Differences in generation times between sisters in 174 two-cell clones.

B differs noticeably from Curve A in the long generation time of 7 hours and the extended period of deceleration. These conditions undoubtedly resulted from the limited availability of oxygen. Both curves clearly indicate the reproducibility of growth response of *Tetrahymena* in 2% proteose peptone broth under controlled conditions.

B. *Growth in small clones.* Single cells which had been isolated from cultures in log phase to either hanging drops or drops in small watch glasses were timed through two divisions. Generations were calculated between the times the sisters actually separated, to the nearest 5-minute intervals. The distribution of generation times is shown in Figure 4, and the separate experiments are summarized in Table I. The over-all range is 2 hours 15 minutes (135 minutes) to 8 hours 25 minutes (505 minutes); the mean generation time is 223 ± 53.0 minutes. This is close to the value of 4 hours (240 minutes) computed from Klett readings of cultures grown in tubes which were aerated on a roller, indicating that the conditions in small drops were favorable for rapid growth.

C. *Generation times of sister cells.* The differences in generation times of sister cells are shown in Figure 5. In contrast to the wide range of generation times among different clones, sisters tend to have very similar generation times, the mean difference being 14 minutes. Rarely did a cell divide more than 30 minutes later than its sister, and often the sisters divided simultaneously (*i.e.*, within the same two-minute interval). The difference between sisters was not correlated with length of generation time. In some clones the two sisters divided almost synchronously after 5 to 7 hours, while some cells with short generation times had sisters which did not divide until an hour or more later.[4]

II. *Nuclear phenomena in the life cycle*

A. *Cytology.* Photographs of several cells from a mass culture in log phase, stained by the Feulgen method and counterstained with fast green, are shown in Figure 6. Macronuclei of *T. pyriformis* H range in diameter from about 5 to 12 μ, with an average size of about 8 μ. Before division starts, the enlarged nucleus appears to consist of strands of chromatin granules wound into a ball (stage 1). The nucleus then elongates, and the strands are pulled out along its length (stage 2). As division proceeds, and the nucleus separates into the two daughters (stage 3), broken ends of strands can sometimes be seen. Part of a strand (or strands) may remain behind in the cytoplasm (stage 4) where it condenses into a small Feulgen-positive body. Similar in appearance to micronuclei, these bodies are not present in all cells, and in time seem to be resorbed by the cytoplasm. One of these particles is visible in the left of the two interphase cells (stage 5). The relatively homogeneous interphase nucleus appears to be made up of fine threads of chromatin.

[4] Separation of sisters and "conditioning" of medium did not seem to affect generation time. About 12 hours after 9 individuals had been isolated to drops of broth in watch glasses, all but one of the cells in each drop resulting from the interim divisions were removed. An hour after these cells had divided, one sister of each pair was placed in a fresh drop of medium. All the pairs of separated sisters divided within 20 minutes of each other—three divided synchronously, five cells in fresh medium divided sooner than their sisters, and one divided later.

In these photographs, cytoplasmic vacuoles are demonstrated by the fast green stain, but the mouths of the organisms are not clearly distinguishable.

B. *Photometric analysis of DNA.* 1. *Amount of DNA per cell in mass cultures.* A sample of cells from a logarithmically growing culture (I) (24 hours after inocu-

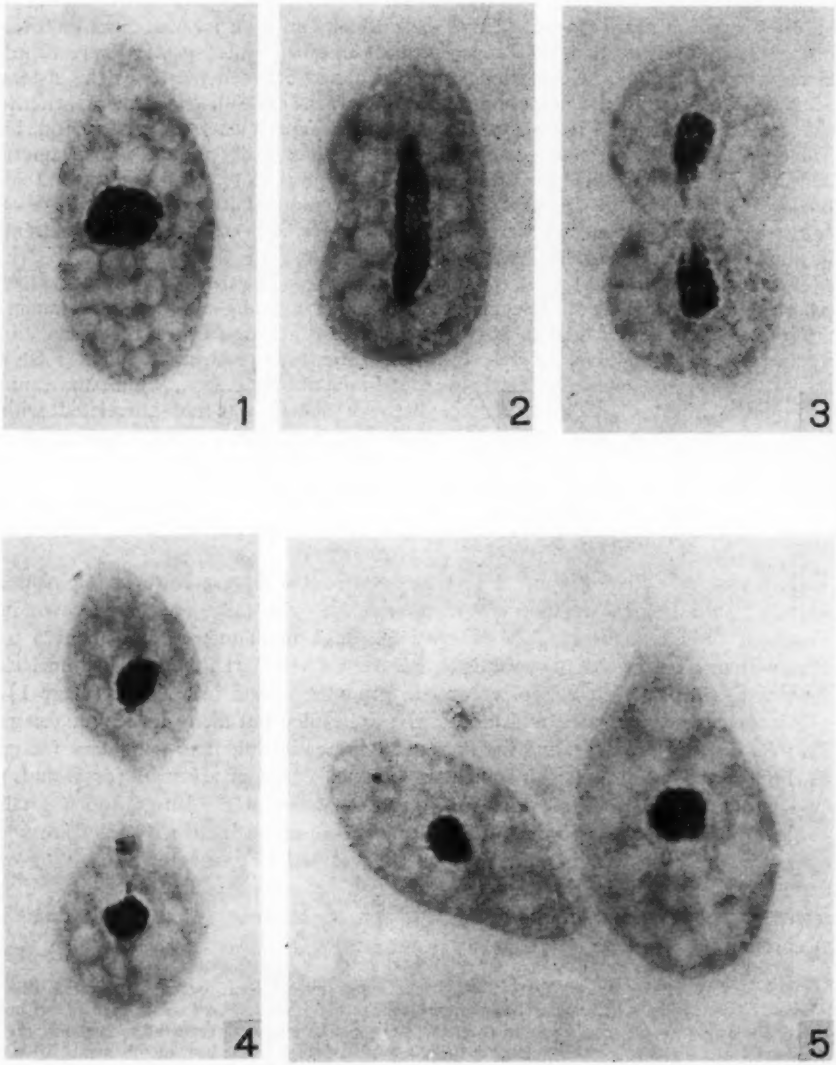

FIGURE 6. Photographs of *Tetrahymena pyriformis* H stained with Feulgen and fast green dyes. Dividing cells in different stages are shown in 1, 2, 3, and 4, and interphase cells in 5. See text for further explanation. Magnification about 1050 ×.

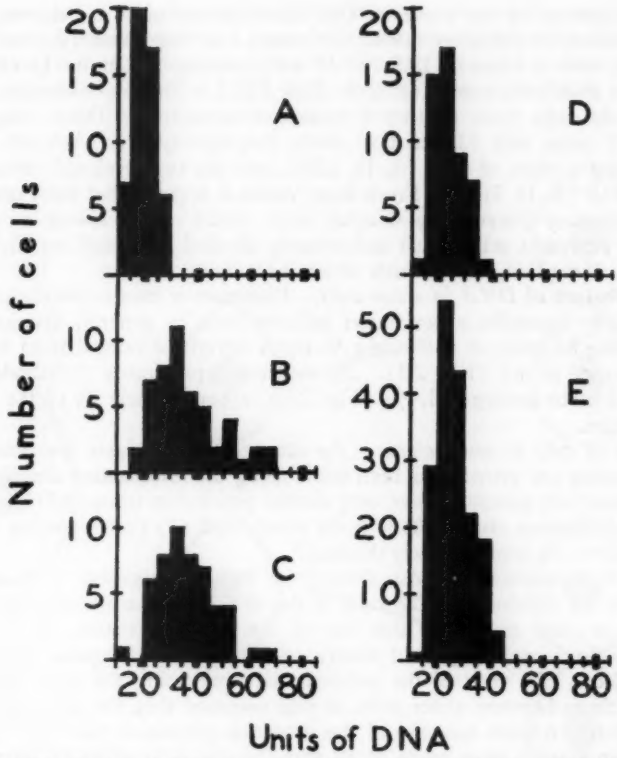

FIGURE 7. Distribution of DNA in mass cultures and isolated cells. (A) Culture I, one day old (log phase). Amount of DNA in 50 dividing daughters (each from a different pair). Range 12.5 to 29.2; mean 19.3 ± 4.35 S. D.; mode 17. (B) Culture I, one week old (stationary phase). Amount of DNA in 50 cells. Range 17.9 to 68.0; mean 35.9 ± 12.9 S. D.; mode 32. (C) Culture I, two weeks old. Amount of DNA in 50 cells. Range 13.9 to 66.6; mean 36.0 ± 11.0 S. D.; mode 32. (D) Culture II, one day old (log phase) (subculture of one-week old Culture I). Amount of DNA in 50 dividing daughters (each from a different pair). Range 13.7 to 36.8; mean 22.8 ± 5.81 S. D.; mode 22. (E) Isolated dividing daughters. Amount of DNA in 173 cells (83 pairs and 7 individuals). Range 12.9 to 43.2; mean 25.7 ± 6.15 S. D.; mode 22.

lation) was fixed and stained. One daughter nucleus of each of 50 dividing pairs (similar to stage 4, Fig. 6) was measured; presumably these values would indicate the basic, or minimal, amount of DNA. (Since the cytoplasm was invisible, criteria for dividing daughters were shape and appearance of nuclei—two rather tear-shaped and granular-looking nuclei, usually with part of a strand of chromatin extending from one toward the other.) One week later this culture, in stationary phase, was sampled again, and 50 nuclei were measured. At the same time, a subculture (II) was inoculated from Culture I. Twenty-four hours later Culture II was sampled, and 50 of its dividing daughters were measured. When Culture I was two weeks old it was sampled again, and 50 more nuclei were measured.

The histograms for the values of DNA in arbitrary units are shown in Figure 7. The amounts in daughter nuclei of Culture I at log phase (A) ranged from 12.5 to 29.2, with a mean of 19.3 (S. D. 4.35); those of Culture II (D) ranged from 13.7 to 36.8, with a mean of 22.8 (S.D. 5.81). The one-week-(B) and two-week-(C) old cells from Culture I contained amounts of DNA ranging from 17.9 to 68.0 units, and 13.9 to 66.6 units, respectively, the week-old stationary culture having a mean of 35.9 (S. D. 12.9), and the two-week-old culture having a mean of 36.0 (S. D. 11.0). From these values it appears that most non-dividing cells in stationary phase have doubled their DNA. The lowest values might conceivably represent cells which had recently divided (although no dividing cells were seen in these samples), or cells which were dying or dead.

2. *Comparison of DNA in sister cells.* Photometric measurements of DNA in pairs of newly separated sister nuclei indicate that, in general, division is quite even. Among 83 pairs of sisters, the % mean difference ranged from 0.1 to 14.2, with an average of 4.7 (Fig. 2B). As mentioned previously (Methods, V), the instrumental error averaged 1.4% (Fig. 2A), approximately ⅓ of the difference between sisters.

3. *DNA in cells in small clones.* An attempt to investigate the time of DNA synthesis during cell growth has been made using the information described above: (1) that sister cells generally have very similar generation times; (2) that distribution of DNA between sister cells is fairly equal; and (3) that following a division, DNA appears to be approximately doubled.[5]

Figure 8 summarizes the plan devised for this investigation, from which data are available for 90 clones. The time of the division of an isolated cell (C) was noted, and at some time after that one of the resulting sisters (C_1) was fixed. When the other sister (C_2) had nearly completed its subsequent division it, in turn, was fixed. In view of the previous data indicating the close similarity of generation times between sister cells, it was assumed that the generation time of C_2 gave a fairly reliable measure of the potential generation time of C_1.[6] Photometric measurements were made of all three macronuclei—that of interphase cell C_1, and those of the two daughters of C_2 (designated C_{2a} and C_{2b}). Adding the DNA in C_{2a} and C_{2b} for the total amount in C_2 indicated the amount of DNA which C_1 would have contained by the time it reached its next division. The ratio of C_1/C_2, then, indicates the relative amount of DNA in C_1 when it was fixed.

[5] Some additional evidence suggesting the duplication of DNA comes from seven pairs of synchronously dividing sisters, each of whose four daughter nuclei were measured photometrically. Among four of these pairs, the two daughter mates with the lower total amount of DNA had 94%, 96%, 97%, and 97% as much DNA as the two higher daughter mates. In the other three pairs, the daughters had become detached, so the mates could not be differentiated. The most extreme possible relative values would have been 82%, 89%, and 96%; the closest possible, 91%, 99%, and 100%.

[6] The generation time of C_2 was known quite closely, although, since dividing cells were fixed, the time of separation had to be estimated by appearance. Cells which are in the process of dividing become quiescent until the division is nearly completed, when they begin swimming, pulling, and twisting. Assuming the time spent dividing to last from the time a cell quiets down and a constriction begins to appear, to the time the daughters actually pull apart, it has been observed to take from about 20 to 35 minutes, regardless of the length of generation time. In these experiments, an average of 25 minutes was used as a basis for calculation. For these cells, the length of interphase time, then, is based on the generation time (known time of separation at the first division to the estimated time of separation at the second division) minus 25 minutes.

If the original cell C had divided its DNA precisely between C_1 and C_2, and no DNA had been synthesized when C_1 was fixed, one would obtain the ratio 0.5. On the other hand, if C_1 had duplicated its DNA by the time it was fixed, one would obtain the ratio 1.0. Ratios of 0.625, 0.75, and 0.875 would indicate, respectively, that 25%, 50%, and 75% of synthesis had occurred.

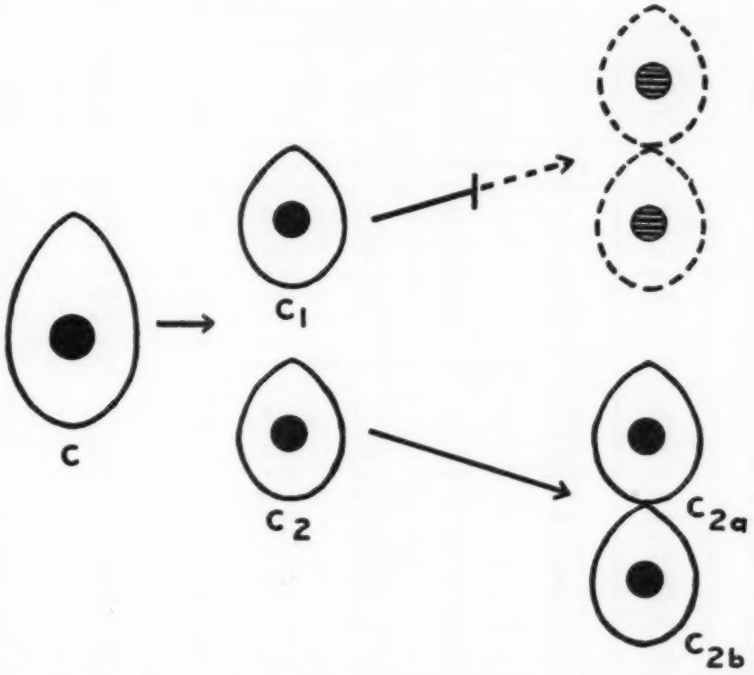

FIGURE 8. Scheme for fixing sister cells. The figure on the left represents an isolated cell (C), in time dividing into the two sister cells in the middle (C_1 and C_2). During the following interphase C_1 was fixed, as indicated by the vertical line, when its DNA might or might not have increased. C_2 was not fixed until its next division, as indicated at bottom right; its generation time was a measure of the potential generation time of its fixed sister, C_1. The total DNA in the C_2 daughters (C_{2a} and C_{2b}) represented the amount which C_1 would have contained at its next division.

In Table II the data are summarized, with the 90 clones grouped according to generation times, and each group arranged according to the age of C_1 when it was fixed. Also included are the period of interphase (see footnote 6) elapsing between the fixation of C_1 and the start of the C_2 division, the relative interphase age of C_1 (age of C_1/length of C_2 interphase), the amounts of DNA in the measured cells, and the relative DNA content of C_1 (C_1 DNA/C_2 DNA). Table I, line 3, summarizes the generation time data for the C_2 cells, and Figure 7E shows the distribution of DNA values for C_{2a} and C_{2b}.

TABLE II

Photometrically measured clones arranged according to generation times

Clone No.	Age C_1 (min.)	Time to next div. (min.)	Age C_1 / Interphase*	Amount of DNA (in arbitrary units)				C_1 DNA / C_2 DNA
				C_1	C_2	C_{2a}	C_{2b}	
Generation time 155–165 minutes								
1	75	60	0.56	44.8	44.3	21.8	22.5	1.01
2	95	35	.73	60.8	58.7	26.7	32.0	1.04
Generation time 170–180 minutes								
3	90	65	.58	53.0	38.6	18.0	20.6	1.37‡
4	95	50	.66	54.3	58.0	28.5	29.5	.94
5	125	25	.83	48.7	54.9	27.2	27.7	.89
6	130	25	.84	62.6	53.6	25.0	28.6	1.17
7	140	15	.90	49.2	49.6	22.7	26.9	.99
8	145	10	.94	51.9	49.0	24.2	24.8	1.06
Generation time 185–195 minutes								
9	30	140	.18	27.1	49.8	23.7	26.1	.54
10	35	130	.21	35.3	57.1	27.9	29.2	.62
11	35	125	.22	23.2	48.0	23.4	24.6	.49
12	50	120	.29	21.6	38.7	19.0	19.7	.56
13	50	110	.31	24.5	32.0†	16.0	—	.77
14	60	110	.35	33.7	34.5	16.5	18.0	.98§
15	65	105	.38	30.7	58.2	26.8	31.4	.53
16	75	85	.47	40.2	51.9	24.4	27.5	.78
17	75	85	.47	64.0	55.0	26.1	28.9	1.16
18	90	80	.53	50.5	45.2	21.0	24.2	1.12
19	120	45	.73	52.0	52.1	25.1	27.0	1.00
20	120	40	.75	47.1	52.1	25.2	26.9	.90
21	155	05	.97	41.4	47.9	22.9	25.0	.86
Generation time 200–210 minutes								
22	30	150	.17	32.1	60.0	29.9	30.1	.53
23	35	150	.19	26.6	43.7	20.6	23.1	.61
24	35	145	.20	27.8	43.4	21.6	21.8	.64
25	45	130	.26	28.0	35.5	17.7	17.8	.79§
26	65	115	.36	33.3	46.8	23.0	23.8	.71
27	90	90	.50	56.6	58.7	29.0	29.7	.97
28	90	85	.51	34.8	48.2†	24.1	—	.72
29	90	85	.51	32.1	40.6	19.6	21.0	.78
30	90	85	.51	36.8	37.3	18.1	19.2	.99
31	95	85	.53	42.6	78.6	38.8	39.8	.54§

* Interphase equals generation time minus 25 minutes.

† Estimate of C_2 DNA based on measurement of one daughter; other daughter unmeasurable.

‡ Unequal division of C obvious from C_1/C_2 ratio.

§ Unequal division of C presumed from exceptional amount of DNA in C_2, and unusual C_1/C_2 ratio for age of C_1.

TABLE II—*Continued*

Clone No.	Age C_1 (min.)	Time to next div. (min.)	Age C_1 / Interphase*	Amount of DNA (in arbitrary units)				C_1 DNA / C_2 DNA
				C_1	C_2	C_{2a}	C_{2b}	
Generation time 200–210 minutes—*Continued*								
32	100	85	.54	34.1	78.9	35.5	43.4	.43§
33	110	70	.61	60.9	63.6	31.5	32.1	.96
34	125	55	.69	53.8	62.6	31.1	31.5	.86
35	130	45	.74	48.8	49.3	23.8	25.5	.99
36	155	20	.89	34.2	33.1	15.7	17.4	1.08
37	160	15	.92	47.5	43.1	19.9	23.2	1.10
38	165	20	.89	40.4	34.4	16.4	18.0	1.17
Generation time 215–225 minutes								
39	70	130	.35	35.8	46.8	23.2	23.6	.77
40	95	100	.49	37.8	61.1	30.0	31.1	.62
41	150	45	.77	42.9	41.6	20.6	21.0	1.03
42	165	30	.85	49.7	47.3	21.7	25.6	1.10
43	165	25	.87	65.7	72.5	35.2	37.3	.91
44	170	30	.85	60.8	71.0	34.8	36.2	.86
45	180	10	.95	33.9	38.4	17.5	20.9	.88
46	185	15	.93	51.1	50.6†	25.3	—	1.01
Generation time 230–240 minutes								
47	35	180	.16	22.3	45.8	22.0	23.8	.49
48	60	150	.29	22.0	59.8†	29.9	—	.37‡
49	65	140	.32	35.6	54.4	25.6	28.8	.66
50	70	135	.34	45.2	40.5	19.4	21.1	1.12
51	140	70	.67	54.7	60.5	27.8	32.7	.91
52	155	55	.74	45.7	45.9	21.1	24.8	1.00
53	180	35	.84	62.7	59.4	29.2	30.2	1.05
54	175	30	.85	59.6	69.6	34.3	35.3	.86
Generation time 245–255 minutes								
55	30	190	.14	19.4	58.9	28.6	30.3	.34‡
56	35	195	.15	24.9	41.7	19.1	22.6	.60
57	60	160	.27	17.6	27.5	12.9	14.6	.64
58	130	90	.57	48.5	64.4	27.6	36.8	.75
59	145	85	.63	41.6	51.6†	25.8	—	.81
60	150	75	.67	43.8	43.8	21.0	22.8	1.00
61	155	65	.71	41.0	37.0	17.5	19.5	1.11
62	160	65	.71	45.7	50.4	25.1	25.3	.91
63	160	60	.73	36.4	47.7	23.5	24.2	.76
64	175	55	.76	46.2	47.8	22.5	25.3	.98
65	190	30	.86	41.0	34.7	17.2	17.5	1.18‡

TABLE II—*Continued*

Clone No.	Age C_1 (min.)	Time to next div. (min.)	Age C_1 / Interphase*	Amount of DNA (in arbitrary units)				C_1 DNA / C_2 DNA
				C_1	C_2	C_{2a}	C_{2b}	
Generation time 260–270 minutes								
66	115	130	.47	29.4	75.5	33.4	42.1	.39‡
67	120	115	.51	41.8	65.1	32.3	32.8	.64
68	145	90	.62	46.8	61.0	29.6	31.4	.77
69	150	85	.64	56.1	65.2	28.7	36.5	.86
70	150	85	.64	47.2	43.8†	21.9	—	1.08
71	155	90	.63	33.2	40.2	18.3	21.9	.83
72	155	80	.66	35.0	34.9	16.1	18.8	1.00
73	200	40	.83	50.0	58.9	28.4	30.5	.85
Generation time 275–285 minutes								
74	65	190	.26	35.3	52.8	24.9	27.9	.66
75	65	185	.26	41.4	69.7	34.8	34.9	.60
76	200	55	.79	42.1	52.0	22.8	29.2	.81
Generation time 290 minutes								
77	120	145	.45	40.0	82.0	38.8	43.2	.49§
Generation time 315 minutes								
78	220	70	.76	69.9	64.2†	32.1	—	1.09
Generation time 320–330 minutes								
79	120	180	.40	30.8	43.7	20.5	23.2	.71
80	135	170	.44	45.0	76.5	37.2	39.3	.59§
81	180	120	.60	48.3	47.6	23.2	24.4	1.01
Generation time 335–345 minutes								
82	150	165	.48	27.2	54.6	26.1	28.5	.50
83	245	70	.78	28.2	33.6	15.5	18.1	.84
Generation time 350 minutes								
84	90	235	.28	27.6	61.3	26.3	35.0	.45
Generation time 365–375 minutes								
85	90	250	.26	26.2	58.5	27.3	31.2	.45
86	180	160	.53	31.0	71.4	35.2	36.2	.43§
87	300	45	.87	35.3	40.5	19.4	21.1	.87

TABLE II—*Continued*

Clone No.	Age C_1 (min.)	Time to next div. (min.)	Age C_1 / Interphase*	Amount of DNA (in arbitrary units)				C_1 DNA / C_2 DNA
				C_1	C_2	C_{2a}	C_{2b}	
Generation time 385 minutes								
88	35	325	.10	16.6	38.3	17.6	20.7	.43
Generation time 405 minutes								
89	35	345	.09	18.2	31.8	15.8	16.0	.57
Generation time 480 minutes								
90	300	155	.66	54.4	43.3	20.3	23.0	1.25‡

The deviations to be expected from the ideal C_1/C_2 ratios of 0.5 and 1.0, before and after synthesis, have been calculated from the DNA values of C_{2a} and C_{2b} nuclei, on the assumption that the parent cells (C) may have divided with the same degree of equality as did their daughters (C_2) (Table III). For these calculations, four ratios were used:

(1) low daughter/high daughter × 2 (the situation to be found if the interphase cell contained the lower amount of DNA and had not started synthesis);
(2) high daughter/low daughter × 2 (if the interphase cell contained the higher amount of DNA and had not started synthesis);
(3) low daughter/high daughter (if the interphase cell contained the lower amount of DNA and had completed synthesis); and
(4) high daughter/low daughter (if the interphase cell contained the higher amount of DNA and had completed synthesis).

Obviously any C_1/C_2 ratios of 0.42 or less, or 1.18 or more, must have resulted from divisions approximately as unequal as the 13 most unequal cases listed in Table III. Table II shows three such low and three such high ratios (marked by symbol ‡). In three of these clones (Nos. 3, 65, 66) the C_2 DNA was considerably above or below the mean (51.4 units) for this group of cells. Partial synthesis, obviously, would tend to obscure other such unequal pairs, and asynchronous growth probably would also be reflected by unusual C_1/C_2 ratios. In 7 cases (marked in Table II with symbol §), however, inequality of DNA content is suggested by exceptionally high or low C_2 values, combined with C_1/C_2 ratios unusual for the age of C_1. A total of 13 presumably unequal pairs among the 90 clones are thus designated—which, remarkably, is also the number of the most unequal of the 83 C_{2a}–C_{2b} cells listed in Table III.

From the remainder of Table III it appears that for the bulk of clones, C_1/C_2 ratios of 0.59 or less indicate that C_1 had synthesized little or no DNA, whereas ratios of 0.85 or more indicate that when C_1 was fixed it probably had completed

duplication of its DNA. Based on these figures, the data from Table II have been summarized in Table IV according to (A) the age of C_1, (B) the interphase time elapsing from the fixation of C_1 to the start of the C_2 division, and (C) the relative interphase age of C_1. In the more detailed examination of the clones, below, the numbers refer to Table II.

Consideration of the early part of interphase shows that, of 11 clones in which the C_1 cells were fixed within the first 30–35 minutes, 7 have C_1/C_2 ratios of less than 0.59, indicating that probably no synthesis had occurred. Ratios for the other four clones (Nos. 10, 23, 24 and 56) are all in the low 0.60's. At the other end of interphase, of 40 clones in which C_1 was fixed 80 minutes or less before the next division began, 37 have C_1/C_2 ratios of 0.85 or more, indicating that in these cases synthesis probably had been completed. Two of the three exceptional clones (Nos. 76 and 83) had C_1/C_2 ratios which were only slightly low (0.81 and 0.84). The other clone, No. 63, had a ratio of 0.76; its C_2 value, however, was not unusually high, which suggests the possibility that asynchronous growth of the sister cells had occurred.

Despite the wide range of interphase times among individual cells, it thus appears that DNA synthesis generally does not begin for a period of perhaps a half hour after the previous division, and is completed a considerable length of time—about 80 minutes—before the next division begins. The process of synthesis, then, would occupy a fairly short period of time in cells with short generation times, and a relatively longer period in cells with long generation times. Presumably C_1 cells fixed during this interim would be in the process of synthesizing DNA. Among 39 such clones, 18 do indeed have C_1/C_2 ratios which indicate that partial synthesis had occurred. Nine others have ratios above 0.85, suggesting that synthesis might have been completed when C_1 was fixed. The other 12 have ratios under 0.59, which appear to indicate that no synthesis had occurred. Six of these clones (Nos. 31, 32, 66, 77, 80, and 86), however, had such high C_2 values as to suggest that

TABLE III

Expected C_1/C_2 ratios of DNA at the beginning (before synthesis) and end (after synthesis) of the interphase period, based upon the distribution of the DNA of C_2 between its daughter cells (C_{2a} and C_{2b})

No. of pairs*	No DNA synthesized		All DNA synthesized	
	$\frac{C_{2a}\dagger}{C_{2b} \times 2}$	$\frac{C_{2b}}{C_{2a} \times 2}$	$\frac{C_{2a}}{C_{2b}}$	$\frac{C_{2b}}{C_{2a}}$
5	0.375–.399	0.667–.626	0.750–.798	1.333–1.252
8	.400–.424	.625–.589	.800–.848	1.250–1.178
21	.425–.499	.588–.556	.850–.898	1.176–1.112
20	.450–.474	.555–.527	.900–.948	1.110–1.054
29	.475–.499	.526–.501	.950–.998	1.052–1.002
83 Total				

* Of the 90 clones in Table II, 7 C_2 cells are represented by a single daughter; hence, only 83 pairs of cells are included in this table.

† In Table II, C_{2a} has been listed as the daughter with the lower value of DNA, and C_{2b} as the daughter with the higher value.

TABLE IV

Ratios of DNA in the 90 clones of Table II arranged in different time sequences

	$\frac{C_1 \text{ DNA}}{C_2 \text{ DNA}}$ ≤0.59	$\frac{C_1 \text{ DNA}}{C_2 \text{ DNA}}$ 0.60–.84	$\frac{C_1 \text{ DNA}}{C_2 \text{ DNA}}$ ≥0.85
	Number of clones		
(A) Age of C_1 when fixed (min.)			
30– 35	7	4	—
40– 65	3	7	1
70– 95	3	5	9
100–135	4	3	7
140–165	1	4	17
170–195	1	—	8
200–225	—	1	2
≥230	—	1	2
(B) Time to C_2 div. (after C_1 fixed) (min.)			
≥235	4	—	—
205–230	—	—	—
175–200	2	4	—
135–170	6	3	1
105–140	4	7	2
85–110	3	8	6
55– 80	—	3	13
25– 50	—	—	18
≤20	—	—	6
(C) $\frac{\text{Age of } C_1}{C_2 \text{ interphase}}$			
0.0–.09	1	—	—
.1–.19	5	2	—
.2–.29	5	6	—
.3–.39	1	4	2
.4–.49	4	3	1
.5–.59	3	4	5
.6–.69	—	3	10
.7–.79	—	3	10
.8–.89	—	—	12
.9–.99	—	—	6

very unequal division of DNA had occurred between the sister cells, thus masking the start of synthesis. In three other cases (Nos. 12, 15, 48), C_1 was fixed so early in interphase (65 minutes or less) that the low ratios probably have little significance. The last three clones (Nos. 82, 84, 85) are unexplained exceptions; possibly the apparent lack of synthesis in C_1 is related to the unusually long generation times.

The data obtained in the course of this study suggest that cells duplicate each unit of DNA during their growth cycles. The presence of 25 clones in the "partial synthesis" group (C_1/C_2 0.60 to 0.84) indicates that the synthesis is not instan-

taneous. In the "completed synthesis" group there are 43 clones with C_1/C_2 ratios of 0.85 to 1.17. (To be sure, some of the low ratios might represent C_1 cells still in the process of synthesis.) Of these 43 clones, 21 have ratios of 0.99 or less (indicating that C_1 contained the lower amount of DNA), 4 have ratios of 1.00, and 18 have ratios above 1.00 (indicating that C_1 contained the higher amount of DNA); selection of the cell with the lower or higher amount of DNA for C_1 was thus perfectly random. In 16 of these clones (37%) the sisters contained nearly equal amounts of DNA (C_1/C_2 ratios of 0.95–1.04); in 12 (28%) the ratios were less equal (0.90–0.94, and 1.05–1.10); and in 15 (35%) the ratios were even less equal (0.85–0.89, and 1.11–1.17). These figures take on special significance when compared with the 70 C_{2a}–C_{2b} cells falling in the same ratio groups (Table III, lines 3, 4, 5)—29 (41%) are in the first group, 20 (29%) in the second, and 21 (30%) in the third. Such close correspondence between these two sets of cells—sisters just at the end of the division process, and sisters of the ensuing life cycle—offers good evidence that, following division, the DNA content of a cell is precisely duplicated.

This investigation of DNA synthesis in *Tetrahymena* indicates, further, that a considerable time elapses from the completion of synthesis to the end of the interphase period. It is intriguing to consider the implications of this time period. Cytological examination shows no obvious, complicated mitotic apparatus being formed, although the chromatin does undergo a fairly regular condensation. Cellular reorganization, however, occurs before the mother cell divides into two daughters. The most striking change which takes place before the start of division is the formation of a new mouth (Furgason, 1940), allotted to the posterior daughter of the dividing pair. By studying growing cells under a phase microscope, it is hoped to determine the amount of time necessary for this process.

Discussion

The synthesis of DNA by *Tetrahymena pyriformis* H clearly occurs during the interphase period. Other workers, in some cases using very different methods of analysis, have come to similar conclusions for cells with mitotically dividing nuclei—the micronuclei of a ciliate (*Chilodonella uncinatus*—Seshachar, 1950), vertebrate cells (Swift, 1950), chick fibroblasts in tissue culture (Walker and Yates, 1952), sea urchin embryos (McMaster, 1955), *Vicia faba* root tips (Howard and Pelc, 1951; Deeley *et al.*, 1957), onion root tips (Patau and Swift, 1953).

After the end of a division (the beginning of the new interphase) a period of time elapses before DNA synthesis begins. This pause was particularly obvious in some of the individual clones with long generation times, when several hours might elapse before synthesis could be detected. Synthesis appears to be completed more than an hour before the cells begin to divide, a considerable period when compared to their average generation time of around four hours.

The exact duplication of DNA during interphase is suggested strongly (1) in mass cultures, by the amounts of DNA in the macronuclei of dividing daughter cells during log phase, as compared with the amounts in older, non-dividing cells, and (2) in individual clones, by the similar DNA ratios in pairs of dividing daughters, as compared with those in sister pairs after synthesis of DNA had occurred.

In general, the amounts of DNA allotted to sister nuclei are remarkably close. Frequently, however, division is quite unequal; furthermore, very often a piece of chromatin is left behind in the cytoplasm. Such irregularities might be expected to result in genic imbalance, and death; instead, they seem to result in the wide range of DNA values found among non-clonal cells. Very occasional deaths have been noted among isolated cells (2 among a set of 101 cells, for example), which might be attributable either to cellular abnormality, or to some external factor. Among cells fixed during growth, occasional diffuse-looking nuclei have been found which were unsuitable for photometric analysis—possibly they represented dying cells, or possibly they resulted from faulty fixation. That unequal division of the macronucleus does not usually have an adverse effect is shown by the fact that the DNA ratios between apparently healthy sister cells, after synthesis of new DNA, are as variable as those between dividing daughters. It seems reasonable, however, that some method for a fairly orderly distribution of chromatin must be present in this micro-organism, with no micronucleus but only an amitotically dividing macronucleus, which has successfully propagated itself for almost 30 years in the laboratory. At division, the appearance of the *Tetrahymena* macronucleus does suggest some sort of organization.

Unfortunately, the basic structure of ciliate macronuclei is difficult to interpret. In *Paramecium aurelia,* however, Sonneborn (1947) obtained genetic evidence (the regeneration of parts of degenerating macronuclei) for genome segregation, from which he concluded that the macronucleus must contain about 40 diploid "subnuclei," distributed at amitotic division in intact units. (It will be recalled that Moses, 1950, estimated the macronucleus of *P. caudatum* to contain about 40 times as much nucleoprotein as the micronucleus.) Kimball (1953) could find no cytological evidence for subunits in the macronucleus of *P. aurelia,* however, but only for a high degree of polyploidy.

Grell, too, has been unable to find cytological evidence for subnuclei in the macronuclei of suctoreans (for example, Grell, 1953b). The budding by which these organisms reproduce vegetatively, however, suggests that genome segregation must occur. Particularly in the free-living stage of *Tachyblaston ephelotensis,* Grell (1950) noted the similarity in size and structure of the parts budded off the parent macronucleus, with its linear arrangement of chromatin (he reported that there appeared to be 8 chromatin elements in each bud). In another type of protozoan—a radiolarian, *Aulacantha scolymantha*—Grell (1953c) has found clear cytological evidence for a method by which genome segregation could occur. In the highly polyploid nucleus of this organism, the chromosomes appear to be linearly arranged in complete, individual sets, forming numerous chains of "Sammelchromosomen." Random separation of these intact genomes necessarily would result in perfectly balanced daughter nuclei. It is possible that the occurrence of "Sammel" chromosomes might also explain the efficiency of amitosis in ciliates, although the division figure of the *Aulacantha* nucleus, which also has no spindle, appears to be quite different from those of ciliate macronuclei.

Purely in the realm of speculation, it has occurred to the present author that, rather than "Sammel" chromosomes, the Feulgen-positive granular strands which appear to extend the length of the dividing macronucleus in *Tetrahymena* might each represent a row of identical chromosomes, held together by forces of attraction. If this were the case, the daughter nuclei would be assured of a fairly well balanced

assortment of chromosomes, no matter how unequal the division (assuming that all the strands separated in approximately the same region). The chromatin fragments left behind at division would probably cause no serious imbalance, and might, indeed, be a way of correcting a nucleus somewhat imbalanced by the previous division. If a similar chromosome arrangement also occurred in the degenerating macronuclear skein of *Paramecium*, the parts breaking off would very likely contain complements of chromosomes, as has been suggested by the regeneration experiments. A condition such as this could also explain the efficiency of the budding of suctoreans.

Another characteristic of *T. pyriformis* H which has been demonstrated in the present experiments is the variability in generation times among different clones, as compared with the usual close similarity between sister cells. The length of generation time seems to have no relation to the amount of DNA.

Once synthesis of DNA has been completed, the photometric measurements of mass culture cells indicate that under some conditions division does not necessarily follow immediately. In general, cells from the one-week- and two-week-old culture contained approximately twice as much DNA as individual dividing daughters from log phase. Prescott's recent studies (1957) of generation time and lag phase in strain HS indicate that cells from stationary phase may divide soon after inoculation into fresh medium, and may then undergo a lag phase before logarithmic growth begins. Such preliminary division seems a reasonable consequence if the inoculated cells contained a doubled amount of DNA.

By alternating temperature changes, Scherbaum and Zeuthen (1954) caused logarithmically growing cells (strain GL) essentially to stop dividing until the final return to optimal temperature. The following synchronous (85%) division occurred 90 ± 10 minutes later, a time period remarkably similar to that found in the present experiments (about 80 minutes) to occur between the end of synthesis and the end of the interphase period. They report that the following two somewhat less synchronous divisions of the treated cells occurred about 1.7 hours (100 minutes) apart. Correlated with these interesting data is the fact that they found (Zeuthen and Scherbaum, 1954) by Hoff-Jørgensen microbiological assay that cells at the end of treatment contained about four times as much DNA as normally growing cells. By Schmitt-Thannhauser analysis, Ducoff (1956) has confirmed the unusual amount of DNA synthesis by temperature-treated cells. In view of the degree of DNA synthesis and the time elapsing before the first synchronous division, the question arises, as in the present experiments, about the length of time required for cellular changes (such as the formation of a new mouth) which must take place before division can begin.

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Summary

1. In a mass culture of *Tetrahymena pyriformis* H which has stopped growing, the macronuclei contain approximately twice as much DNA as do newly divided macronuclei in a logarithmically growing culture.

2. Non-clonal cells show considerable variability as to DNA content and generation time.

3. Cells in small clones show close similarity as to DNA content and generation time.

4. Duplication of DNA occurs during an intermediate part of interphase, starting some time after the end of the previous cell division, and reaching completion a considerable period of time before the next division begins.

LITERATURE CITED

ALFERT, M., AND I. I. GESCHWIND, 1953. A selective staining method for the basic proteins of cell nuclei. *Proc. Nat. Acad. Sci.*, **39**: 991–999.

ALFERT, M., AND N. O. GOLDSTEIN, 1955. Cytochemical properties of nucleoproteins in *Tetrahymena pyriformis;* a difference in protein composition between macro and micronuclei. *J. Exp. Zool.*, **130**: 403–421.

CHEN, T. T., 1940. Conjugation in *Paramecium bursaria* between animals with very different chromosome numbers and between animals with and without micronuclei. *Proc. Nat. Acad. Sci.*, **26**: 243–246.

CHEN, T. T., 1944. Staining nuclei and chromosomes in protozoa. *Stain Tech.*, **19**: 83–90.

CORLISS, J. O., 1953. Comparative studies on holotrichous ciliates in the *Colpidium-Glaucoma-Leucophrys-Tetrahymena* group. II. Morphology, life cycles and systematic status of strains in pure culture. *Parasitology*, **43**: 49–87.

DEELEY, E. M., H. G. DAVIES AND J. CHAYEN, 1957. The DNA content of cells in the root of *Vicia faba*. *Exp. Cell Res.*, **12**: 582–591.

DI STEFANO, H. S., 1948. A cytochemical study of the Feulgen nucleal reaction. *Chromosoma*, **3**: 282–301.

DUCOFF, H. S., 1956. Radiation-induced fission block in synchronous cultures of *Tetrahymena pyriformis* W. *Exp. Cell Res.*, **11**: 218–220.

ELLIOTT, A. M., AND R. E. HAYES, 1953. Mating types in *Tetrahymena*. *Biol. Bull.*, **105**: 269–284.

FURGASON, W., 1940. Significant cytostomal pattern of "*Glaucoma-Colpidium* group" and a proposed new genus and species, *Tetrahymena geleii*. *Arch. f. Protist.*, **94**: 224–266.

GRELL, K. G., 1950. Der Generationswechsel des parasitischen Suktors *Tachyblaston ephelotensis* Martin. *Zeitschr. f. Parasit.*, **14**: 499–534.

GRELL, K. G., 1953a. Die Konjugation von *Ephelota gemmipara* R. Hertwig. *Arch. f. Protist.*, **98**: 287–326.

GRELL, K. G., 1953b. Die Struktur des Makronucleus von *Tokophrya*. *Arch. f. Protist.*, **98**: 466–468.

GRELL, K. G., 1953c. Die Chromosomen von *Aulacantha scolymantha* Haeckel. *Arch. f. Protist.*, **99**: 1–54.

HETHERINGTON, A., 1933. The culture of some holotrichous ciliates. *Arch. f. Protist.*, **80**: 255–280.

HOWARD, A., AND S. R. PELC, 1951. Nuclear incorporation of P^{32} as demonstrated by autoradiographs. *Exp. Cell Res.*, **2**: 178–187.

KIDDER, G. W., V. C. DEWEY AND M. R. HEINRICH, 1954. The effect of non-ionic detergents on the growth of *Tetrahymena*. *Exp. Cell Res.*, **7**: 256–264.

KIMBALL, R. F., 1953. The structure of the macronucleus of *Paramecium aurelia*. *Proc. Nat. Acad. Sci.*, **39**: 345–347.

MCMASTER, R. D., 1955. Desoxyribose nucleic acid in cleavage and larval stages of the sea urchin. *J. Exp. Zool.*, **130**: 1–27.

MOSES, M. J., 1950. Nucleic acids and proteins in the nuclei of *Paramecium*. *J. Morph.*, **87**: 493–536.

NANNEY, D. L., 1953a. Mating type determination in *Paramecium aurelia*, a model of nucleocytoplasmic interaction. *Proc. Nat. Acad. Sci.*, **39**: 113–119.

NANNEY, D. L., 1953b. Nucleocytoplasmic interaction during conjugation in *Tetrahymena*. *Biol. Bull.*, **105**: 133–148.

NANNEY, D. L., 1956. Caryonidal inheritance and nuclear differentiation. *Amer. Nat.*, **90**: 291–307.

NANNEY, D. L., 1957. The role of cytoplasm in heredity. *In:* A Symposium on the Chemical Basis of Heredity, W. D. McElroy and B. Glass, eds., The Johns Hopkins Press, Baltimore, 134–164.

NANNEY, D. L., AND P. A. CAUGHEY, 1953. Mating type determination in *Tetrahymena pyriformis*. *Proc. Nat. Acad. Sci.*, **39**: 1057–1063.

ORNSTEIN, L., 1952. The distributional error in microspectrophotometry. *Lab. Invest.*, **1**: 250–265.

PATAU, K., 1952. Absorption microphotometry of irregular-shaped objects. *Chromosoma*, **5**: 341–362.

PATAU, K., AND H. SWIFT, 1953. The DNA content (Feulgen) of nuclei during mitosis in a root tip of an onion. *Chromosoma*, **6**: 149–169.

POLLISTER, A. W., 1952. Photomultiplier apparatus for microspectrophotometry of cells. *Lab. Invest.*, **1**: 106–114.

PRESCOTT, D. M., 1957. Change in physiological state of cell population as a function of culture growth and age (*Tetrahymena geleii*). *Exp. Cell Res.*, **12**: 126–134.

SCHERBAUM, O., AND E. ZEUTHEN, 1954. Induction of synchronous cell division in mass cultures of *Tetrahymena pyriformis*. *Exp. Cell Res.*, **6**: 222–227.

SESHACHAR, B. R., 1950. Desoxyribonucleic acid content of the ciliate micronucleus. *Nature*, **165**: 848.

SESHACHAR, B. R., AND C. M. S. DASS, 1953. Macronuclear regeneration in *Epistylis articulata*. *Quart. J. Micr. Sci.*, **94**: 185–192.

SONNEBORN, T. M., 1939. *Paramecium aurelia:* mating types and groups; lethal interactions; determination and inheritance. *Amer. Nat.*, **73**: 390–413.

SONNEBORN, T. M., 1947. Recent advances in the genetics of *Paramecium* and *Euplotes*. *Adv. in Genetics*, **1**: 263–358.

SONNEBORN, T. M., 1951. Some current problems of genetics in the light of investigations on *Chlamydomonas* and *Paramecium*. *Cold Spring Harb. Symp. Quant. Biol.*, **16**: 483–503.

SONNEBORN, T. M., 1953. Patterns of nucleocytoplasmic integration in *Paramecium*. *Proc. 9 Intern. Congr. Genetics, Caryologia*, **6** (Suppl. I): 307–325.

SONNEBORN, T. M., 1954. Is gene K active in the micronucleus of *Paramecium*? *Microb. Genet. Bull.*, **11**: 25–26.

SWIFT, H., 1950. The desoxyribose nucleic acid content of animal nuclei. *Physiol. Zoöl.*, **23**: 169–198.

SWIFT, H., 1953. Quantitative aspects of nuclear nucleoproteins. *Int. Rev. Cyt.*, **2**: 1–76.

WALKER, P. M. B., AND H. B. YATES, 1952. Nuclear components of dividing cells. *Proc. Roy. Soc. London, Ser. B*, **140**: 274–299.

WICHTERMAN, R., 1953. The Biology of Paramecium. The Blakiston Co., New York.

ZEUTHEN, E., AND O. SCHERBAUM, 1954. Synchronous divisions in mass cultures of the ciliate protozoan *Tetrahymena pyriformis* as induced by temperature changes. *In:* Recent Developments in Cell Physiology, J. A. Kitching, ed.; Proceedings of the 7th Symposium of the Colston Research Society, London, 141–157.

CONTRACTILE PROTEIN FROM CRAYFISH TAIL MUSCLE

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Physicochemical studies on vertebrate striated muscle clearly indicate that the interaction of actomyosin, the muscle contractile protein, with adenosine triphosphate (ATP) may be the fundamental phenomenon on the molecular level in muscular contraction (*cf.* Szent-Györgyi, 1951; Weber and Portzehl, 1954). More and more evidence to support this thesis has been obtained in the field of comparative biochemistry in the animal kingdom (Maruyama and Tonomura, 1957).

In invertebrates, the highly developed muscles of arthropods and molluscs have been investigated biochemically in detail. In arthropods, however, there are very few reports on crustacean contractile protein, whereas the characteristics of insect actomyosin have been well established (Gilmour and Calaby, 1953; Maruyama, 1954, 1957b, 1957c). Edsall and Mehl (1940) investigated flow birefringence properties of lobster myosin in relation to the protein denaturation. Humphrey (1948) prepared myosin from the crab, *Maia*, and briefly described its adenosinetriphosphatase (ATPase) properties. Shrinkage of the glycerine-treated muscle fibers of *Limulus* with ATP was observed by Sarkar (1950). On the physicochemical properties of crustacean tropomyosin, detailed works have been recently published (Tsao, Tan and Peng, 1956; Laki, 1957; Kominz, Saad and Laki, 1957).

The present article is concerned with the results of a comparative biochemical study on the ATP-myosin B system and several associated enzymes in crayfish tail muscle.

Materials and Methods

Materials. The crayfish, *Cambarus clarkii*, was used as material. Tail muscles were carefully isolated from exoskeleton and well washed with cold de-ionized water. In a few cases, jaw muscles were also dissected out.

Preparation of contractile protein. The so-called myosin B or natural actomyosin was extracted and purified as established in rabbit skeletal muscle (Szent-Györgyi, 1945). Muscles were homogenized in ten times the volume of cold 0.05 *M* KCl in a Waring Blendor and the water-extractable portion was removed by centrifugating at 3000 G for 5 minutes at 0° C. The residue was washed twice more and finally suspended in five times the volume of the Weber-Edsall solution (0.6 *M* KCl, 0.04 *M* $NaHCO_3$, 0.01 *M* Na_2CO_3). After being placed for 24 hours at 0° C., the suspension turned so viscous that it was diluted with an equal volume of 0.6 *M* KCl. The suspension was centrifuged for 10 minutes to remove the insoluble matter. The viscous supernatant was neutralized to pH 6.5–6.8 with dilute acetic acid and diluted in ten times the volume of cold de-ionized water. The flocculant precipitate was collected by centrifugation, dissolved in 0.6 *M* KCl and diluted in water. This dilution-precipitation procedure was repeated three times

and finally the substances insoluble in 0.6 *M* KCl were removed by centrifugation at 8000 G for 30 minutes at 0° C.

Methods of assaying physicochemical properties. The measurements of ultraviolet absorption spectra were carried out with a Shimazu spectrophotometer. Purine and pentose contents were estimated according to Schneider's procedure (Schneider, 1946). Solubility in KCl was tested at pH 7.0 at 0° C., as described in an earlier paper (Maruyama, 1957a). Salting-out analysis was carried out according to Snellman and Tenow (1954), as modified by Tonomura and his associates (1956).

Methods of observing physical changes with ATP. Super-precipitation was observed in a test tube, containing 0.03 *M* tris-(hydroxymethyl)-aminomethane (Tris) buffer, 1 mM ATP and 1.5–2.5 mg. protein at pH 7.0 and 25° C. Viscosity was measured with viscosimeters of the usual Ostwald type at pH 6.4 at 5–10° C. Turbidity was determined in a Hitachi turbidimeter at pH 6.4 and 15° C. For investigation of viscosity and turbidity, the concentration of myosin B was suitably adjusted by dilution with 0.6 *M* KCl. A small amount of concentrated ATP was added to test the ATP effect.

Enzyme tests. The ATPase activity was determined by measuring the increase in inorganic phosphorus (P) after a specified time, usually 5 minutes at 30° C., in a system containing the protein dissolved in 0.6 *M* KCl, 1 mM ATP, 0.033 *M* Tris buffer (pH 7.0), 0.6 *M* KCl, and 10 mM $CaCl_2$ or some other ions, as specified. Total volume of the reaction mixture was 1.5 ml. In order to investigate the effect of pH of incubation, 0.05 *M* histidine was substituted for Tris. The reaction was started by the addition of substrate and stopped by the addition of 0.5 ml. of 20% trichloroacetic acid (TCA). Appropriate blanks were always run simultaneously with the experimentals.

The water-extractable apyrase activity was determined on the 0.05 *M* KCl extract of muscle suspensions. On proving the occurrence of adenylate kinase in crayfish muscle, the 0.05 *M* KCl extract was treated with heat and acid according to Colowick and Kalckar (1943) and incubated in the ATPase-assaying system with and without crayfish myosin B. Adenylate deaminase activity was tested as described before (Maruyama, 1957a).

ATP was purchased as crystalline disodium salt from Sigma and AMP from Schwarz Labs.

Protein was estimated by multiplying nitrogen values, obtained by a micro-Kjeldahl procedure, by the factor 6.

Inorganic phosphorus was measured on a 1.0-ml. aliquot of the TCA supernatant by a micro-modification of the method of Lohmann and Jendrassik (1926), as described by Moriwaki (1956).

Results

Some Physicochemical Properties

Absorption spectra. Ultraviolet absorption spectra of crayfish myosin B dissolved in 0.6 *M* KCl showed the characteristics of protein nature: a maximal absorption was found around 275 mμ and a minimal one was at 255 mμ. Extinction coefficient (ϵ) on basis of gram N per *l*, and 1.0 cm. light path was 9.0 at 275 mμ,

which was somewhat higher than that of rabbit myosin B (Tarver and Morales, 1951). The ratio of absorption coefficient at 275 mμ to that at 255 mμ is 1.3.

Purine and pentose contents. The results described above suggest that some minute amounts of purine-containing substances such as nucleotides or nucleic acids were present as contaminants in the preparations. The acid-soluble nucleotide fraction contained 1.5×10^{-5} moles purine per gram myosin B and the nucleic acid fraction contained 1.0×10^{-5} moles. The determinations of pentose showed approximately equimolar amounts and any detectable amounts of desoxyribose were not present. These values are a little higher than those for rabbit myosin B (Buchthal *et al.*, 1951).

Solubility in KCl. Crayfish myosin B was completely soluble in concentrations of KCl higher than 0.4 *M*. The solubility curve is quite in accord with that of rabbit myosin B (Szent-Györgyi, 1945). Myosin B dissolved in 0.6 *M* KCl was slightly yellowish white in color.

TABLE I

Effect of varied concentrations of Ca, Mg and EDTA on the super-precipitation of crayfish myosin B

Concn. *M*	Ca	Mg	EDTA
0	++	++	++
10^{-6}	++	++	++
10^{-5}	++	+++	+
10^{-4}	++	±	±
10^{-3}	+	−	−
10^{-2}	±	−	−

Observed within three minutes after the addition of 1 mM ATP at pH 7.0 and 20° C., in the presence of 0.10 *M* KCl.

+++, ++: intense; +: moderate; ±: weak; −: negative.

Salting-out analysis. Under experimental conditions similar to those of Snellman and Tenow (1954), nearly all the proteins were precipitated between 30–38% of saturated ammonium sulfate in the present preparation.

Physical Changes with ATP

Super-precipitation. Under the optimal conditions, *e.g.*, at 20° C. and pH 7.0 in the presence of 0.10 *M* KCl, a typical super-precipitation was found to take place within one minute after the addition of 1 mM ATP, and to reach the end within five minutes. The super-precipitation was evidently recognized in the presence of KCl in concentrations between 0.06 and 0.16 *M*. The optimal KCl concentrations were 0.10–0.12 *M*, which were quite in accordance with those of rabbit myosin B (Szent-Györgyi, 1945). The effects of Ca, Mg and ethylenediaminetetraacetic acid (EDTA), were tested (Table I). These agents in a high concentration (10 mM) inhibited the super-precipitation. Calcium ions affected little, having no accelerating effect. Magnesium ions greatly speeded up the precipitation in 10^{-5} *M*, but retarded in over 10^{-4} *M*. On the other hand, EDTA inhibited in concentrations higher than 10^{-4} *M*. It should be noted that the inhibitory effects of high concentrations of Mg or Ca and of EDTA were qualitatively different: after several hours, the precipitation took place even in the presence of a high concentration of Mg or Ca, but in the presence of EDTA no precipitation was observed to occur.

Change of viscosity. The relative viscosity of crayfish myosin B dissolved in 0.6 *M* KCl was highly anomalous and ATP caused a marked drop in the viscosity (Fig. 1).

According to Weber and Portzehl (1952), the extent of the viscosity change of actomyosin with ATP (ATP sensitivity) can be expressed as follows:

$$\text{ATP sensitivity} = \frac{Z\eta - Z\eta_{ATP}}{Z\eta_{ATP}} \times 100$$

Here $Z\eta = 1n\eta rel/C$; ηrel = relative viscosity in the absence of ATP; $Z\eta_{ATP}$ = that in the presence of a sufficient amount of ATP to cause the maximal viscosity change; and C = concentration of protein (grams/liter). The viscosity data derived from Figure 1 are as follows: $Z\eta = 0.40$, $Z\eta_{ATP} = 0.20$ and the ATP sensitivity = 100%.

The viscosity of myosin B solution drops rapidly with the addition of ATP and gradually rises again when ATP is split by the ATPase action of myosin B. The recovery process followed an S-shaped curve and the recovered viscosity reached about 50% of the drop. Calcium ions, which activate the ATPase action, strongly accelerated the recovery process, while magnesium, an inhibitor of the ATPase action, retarded it. These tendencies are in good accord with those in rabbit or insect myosin B (*cf.* Mommaerts, 1948; Maruyama, 1957b). On the other hand, EDTA, in 10 mM, completely inhibited the viscosity change with ATP.

Change of turbidity. On addition of ATP, the apparent turbidity of myosin B solution decreases because of the decrease of intensity of scattered light (*cf.* Tonomura, 1956). Figure 2 shows the change of turbidity in crayfish myosin B, which

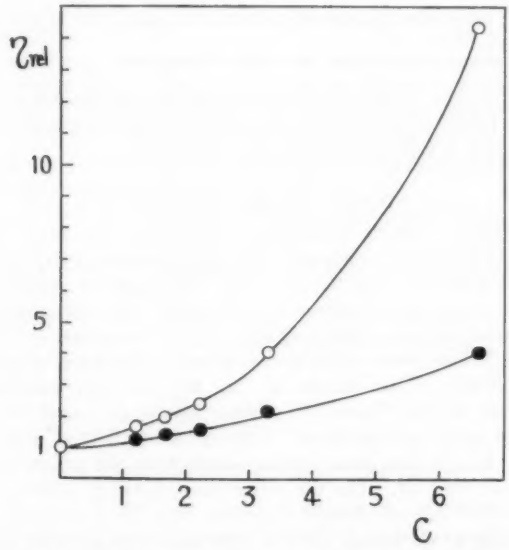

FIGURE 1. Drop of viscosity of crayfish myosin B with ATP; pH 6.4; 6° C.; 0.6 *M* KCl. ○, control; ●, 1 mM ATP added. C, protein concentration, g./*l*.

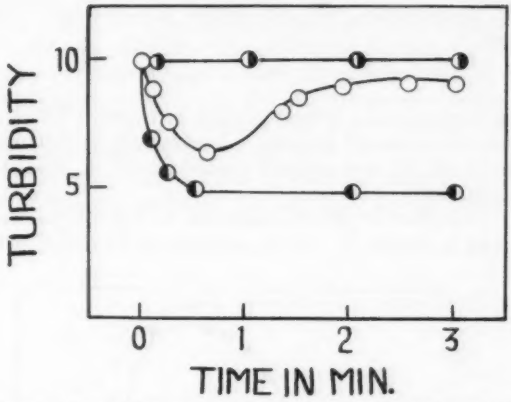

FIGURE 2. Change of turbidity of crayfish myosin B with ATP; pH 6.4; 15° C.; 1 mM ATP; 0.6 *M* KCl. ◑, 10 mM EDTA; ○, 10 mM $CaCl_2$; ◐, 10 mM $MgCl_2$. Turbidity is given as relative level. Time, after the addition of ATP.

is qualitatively similar to that of viscosity described above. The maximal drop of turbidity reached 50% of the original level.

Enzyme Activity

Water-extractable Apyrase

Muscle contains at least two kinds of ATP-splitting enzymes, one myosin itself and the other the water-extractable, magnesium-activated enzyme(s). In crayfish muscle, there was found the latter enzyme(s), too. This fact was already suggested by the classical work of Lohmann (1935) on lobster muscle. The apyrase activity was greatly activated by Mg and completely inhibited by Ca ions. For example, the water-extract, containing 0.3 mg. protein, liberated the following amount of P (μg.) from 2.4 μmole ATP in five minutes at pH 7.0 and 30° C.: 7.0 (none added); 22.0 (5 mM $MgCl_2$); 3.0 (5 mM $CaCl_2$); 2.5 (5 mM $MgCl_2$ plus 5 mM $CaCl_2$). The water-extract of crayfish muscle easily hydrolyzed the two phosphate groups from ATP in the presence of Mg ions.

Adenylate Kinase

In the water-extract of crayfish muscle, adenylate kinase was found to exist. The heat- and acid-treated sample showed no ATPase activity, but in the presence of myosin B, ATPase hydrolyzed the two phosphate groups from ATP. In the presence of 10 mM EDTA, only the terminal phosphate group was set free, even in the combined action of myosin B and the extract. This may be due to the inhibition of adenylate kinase by EDTA, as is well known in rabbit or rat muscle (*cf.* Bowen and Kerwin, 1954).

Adenylate Deaminase

No 5-adenylic acid deaminase activity was detected in crayfish myosin B under the present experimental conditions. This absence of adenylate deaminase activity

in crayfish myosin B coincides with the results of Lohmann (1935) on lobster muscle homogenates.

ATPase

Crayfish myosin B possessed a typical calcium-activated ATPase activity, as well as myosin B's from other animal muscles. Preliminary experiments showed that the ATPase action at pH 7.0 was optimal around 30–35° C. and at 37° C. the enzyme was soon inactivated.

Effect of pH. The effect of pH of incubation on the ATPase action of crayfish myosin B is indicated in Figure 3. In the presence of 10 mM $CaCl_2$ in addition to

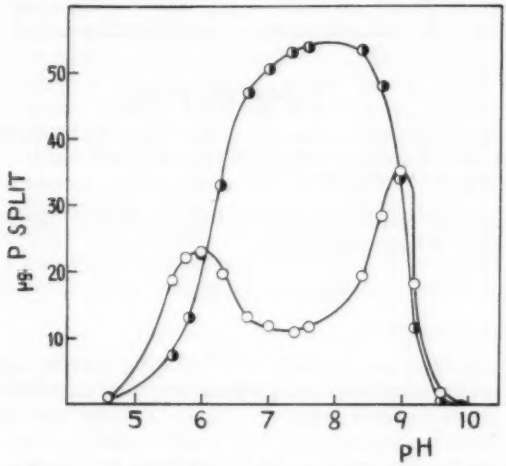

FIGURE 3. Curve of pH-activity of crayfish myosin B ATPase; 0.05 *M* histidine buffer, 30° C.; 5 min. incubation; 0.62 *M* KCl; 2.4 μM ATP; 0.16 mg. protein. ○, 10 mM $CaCl_2$; ◑, 10 mM EDTA.

0.62 *M* KCl and 0.05 *M* histidine, two pH optima were evident: a higher one, at pH 9.0 and a lower one at pH 6.0. In the presence of 10 mM EDTA, a striking activator, a flat, but very high pH optimum between 7–8.5 was found (Fig. 3). The activity level, expressed in terms of Qp (Engelhardt, 1946) is approximately as follows: 1300 at pH 6.0, 600 at pH 7.0 and 2000 at pH 9.0 in the presence of 10 mM $CaCl_2$ and 0.6 *M* KCl at 30° C.; more than 3000 at pH 7–8 in the presence of 10 mM EDTA and 0.6 *M* KCl at 30° C. These values are of a similar magnitude to those reported in actomyosin ATPase from other animals (*cf.* Maruyama and Tonomura, 1957).

Effect of enzyme concentration and incubation time. The enzyme activity was found to be linear to enzyme concentration and incubation time, when the hydrolysis of the substrate surpassed no more than half of the added amount. As is seen in Figure 4, it is apparent that in the presence of 10 mM Ca or EDTA, no phosphate was set free after one-half of the labile phosphates corresponding to the terminal phosphate level of ATP were hydrolyzed. In the presence of Mg ions, the activity

proceeded at a slight, but constant, rate up to 60 minutes. It is clear that crayfish myosin B exhibits a true ATPase action as well as established in the rabbit.

Effect of Ca, Mg and EDTA. The effects of K, Mg, Ca and EDTA on the ATPase activity of crayfish myosin B were investigated in a systematic way. In dilute KCl concentrations of KCl, *e.g.*, 0.1 *M*, the enzyme action was found to be irregular, possibly owing to the remarkable super-precipitation of the protein. However, it is at least sure that in the presence of 0.1 *M* KCl, $MgCl_2$ at 10^{-5} *M* inhibited the ATPase action, at 10^{-3} *M* it elevated the enzyme activity to the original level and at 10^{-2} *M* it again decreased the activity. In the presence of 0.6 *M* KCl,

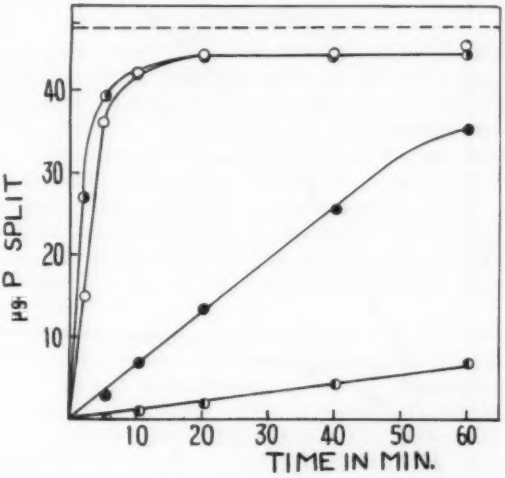

FIGURE 4. Time-activity curve of crayfish myosin B ATPase; pH 7.0; 30° C.; 0.52 *M* KCl; ATP = 48 μg. 1/2 △7P (level of dotted line); 0.5 mg. protein. ●, none added; ◐, 10 mM $MgCl_2$; ○, 10 mM $CaCl_2$; ◑, 10 mM EDTA.

magnesium depressed the ATPase activity increasingly as the concentration became higher from 10^{-5} to 10^{-2} *M*. On the other hand 0.01–0.1 *M* $CaCl_2$ activated ten times the enzyme action, irrespective of the KCl concentration. Mg competitively inhibited the activating effect of Ca.

The accelerating effect of EDTA was most remarkable in the presence of 0.6 *M* KCl; 0.6 *M* NaCl could not substitute for KCl. EDTA was inactive in 0.1 mM and gave the strong activation in concentrations higher than 0.5 mM and optimal in 10 mM (see Fig. 5). The effects of Ca, Mg and EDTA on the ATPase activity are seen in Figure 4.

Effect of substrate concentration. The effect of increasing substrate concentration is summarized in Figure 5. The enzyme reaction proceeded according to the Michaelis-Menten theory:

$$\frac{1}{v} = \frac{1}{Vm} + \frac{Km}{Vm}\frac{1}{S}$$

Here v = reaction velocity of first order, Vm = maximal reaction rate, Km = Michaelis constant and S = substrate concentration, Michaelis constant (Km) was approximately 5×10^{-5} *M* (in the presence of 10 mM Ca or 0.1 mM EDTA), 3×10^{-4} *M* (1 mM EDTA) and 1×10^{-3} *M* (10 mM EDTA). Maximal velocity (Vm) also became much greater in the presence of EDTA, as the concentra-

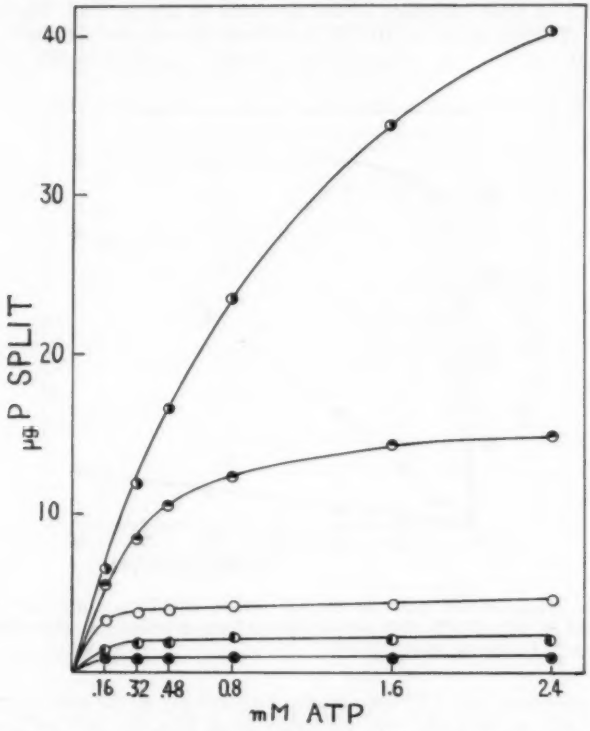

FIGURE 5. Effect of substrate concentration on the ATPase activity of crayfish myosin B; pH 7.0; 30° C.; 3 min. incubation; 0.52 *M* KCl; 0.16 mg. protein. ●, none added; ○, 10 mM $CaCl_2$; ◐, 0.1 mM EDTA; ◓, 1 mM EDTA; ◑, 10 mM EDTA.

tion increased. In the presence of 10 mM Ca ions, Km changed little, although Vm appreciably increased. These facts are in good harmony with those in the rabbit (Tonomura, Matsumiya and Kitagawa, 1957).

Effect of SH reagents. The ATPase activity of crayfish myosin B was very sensitive to heavy metals such as zinc or copper. Especially $ZnSO_4$ in 1 mM completely inactivated the enzyme action. The poisoning action of heavy metals was thought to be mediated through SH groups of myosin B, so that the effect of P-chloromercuribenzoate (PCMB), the powerful SH-blocking agent, was tested; 10^{-4} *M* PCMB nullified the ATPase activity without any pretreatment. After myosin

B was deactivated on a few minutes' pretreatment with 10^{-4} *M* PCMB, glutathione (GSH) was added and after 5 minutes at 20° C., the ATPase action was started by the addition of ATP. GSH in 10^{-3} *M* recovered 10% of the original activity and in 10^{-2} *M* 35% of that was restored. On the other hand, PCMB in a dilute concentration of $5–10 \times 10^{-6}$ *M* rather enhanced the ATPase activity by some 10–30% in the presence of 10 mM Ca as activator. The latter fact confirms the recent finding of Kielley and Bradley (1956) on rabbit myosin ATPase. These influences of PCMB and GSH show that SH groups may be involved in the active center of the ATPase action in the protein molecule.

Discussion

From the observations described above, the interaction of myosin B from crayfish tail muscle with ATP is quite the same as that of rabbit myosin B. The great extent of physical changes, e.g., super-precipitation, drop of viscosity or of turbidity with ATP, shows the main component of the present myosin B preparation was actomyosin. The salting-out analysis suggested that most of the proteins might be actomyosin and a minor part of them be myosin. There is no evidence to show the presence of tropomyosins as contaminants in the preparation. The thread prepared from crayfish myosin B contracted on addition of ATP. It is to be noted that myosin B from crayfish jaw muscle was very similar to that from tail muscle with respect to the ATPase activity, and super-precipitation and viscosity change with ATP.

Attention should be arrested to the pH optima of the ATPase activity of crayfish myosin B. The pH-activity curve in the presence of 10mM $CaCl_2$ and 0.6 *M* KCl is quite similar to that of molluscan myosin B (Maruyama, 1957a), not to that of insects (Maruyama, 1954). This is rather of the vertebrate type (*cf.* Engelhardt, 1946). In any case, two pH optima in the presence of Ca, activation by Ca and inhibition of the Ca effect by Mg in the ATPase action are all common to myosin B's from higher invertebrate and all vertebrate muscles so far reported (*cf.* Maruyama and Tonomura, 1957), although the former seems not to be the case in myosin ATPase from squid mantle muscle, where only the acid optimum was observed (de Villafranca, 1955).

Although essentially the same regards with the interaction between the contractile protein and ATP, one definite difference exists between actomyosins from invertebrate and vertebrate muscles. This is the absence of adenylate deaminase activity in invertebrate actomyosins. This fact was first discovered in insect muscle by Gilmour and Calaby (1953) and later confirmed in insects (Maruyama, 1957c), a sea-anemone (Maruyama, 1957d) and molluscs (Kitagawa and Tonomura, 1957; Maruyama, 1957a). These comparative studies may throw a strong doubt on the essential physiological role of adenylate deaminase in muscular activity. From the viewpoint of biochemical evolution, it is of much interest to investigate whether adenylate deaminase activity is present in prochordate muscles, especially in Acrania.

The relatively large quantity of easily available muscle of crayfish tail will make it a highly desirable material for a further study of the biochemistry and biophysics of the muscle contractile proteins from the comparative point of view. Crayfish myosin B was shown to possess the most similar peculiarities to rabbit in invertebrates so far known (*cf.* Maruyama and Tonomura). Especially the role of

the guanidine kinase system in the recovery process of the ATP-induced physical change of the actomyosin solution, suggested by Tonomura's school (1956) in *Pecten* adductors, is expected to be elucidated more distinctly in crayfish tail muscle.

Summary

1. Contractile protein (myosin B) was extracted and purified from tail muscle of the crayfish *Cambarus clarkii*.

2. Ultraviolet absorption spectra of crayfish myosin B dissolved in 0.6 *M* KCl showed a protein nature. The $\epsilon 275$ was 9 and $\epsilon 275/\epsilon 255$ was 1.3. Acid-soluble purine-pentose content was 1.5×10^{-5} mole per gram protein and nucleic acid purine-pentose content was 1×10^{-5} mole.

3. Crayfish myosin B was quite soluble in 0.4 *M* KCl. Salting-out analysis with ammonium sulfate indicated the main component is actomyosin.

4. Super-precipitation with ATP was clearly seen to take place in the range of 0.08–0.16 *M* KCl concentrations. The phenomenon was most distinguished in the presence of 0.1 *M* KCl and 10^{-5} *M* $MgCl_2$ at pH 7.0 at 25° C. EDTA, in concentrations higher than 10^{-4} *M*, completely inhibited the precipitation.

5. A drop of viscosity with ATP was observed in the presence of 0.6 *M* KCl. The ATP sensitivity of Weber and Portzehl was 100%. The recovery process was also observed to a considerable extent.

6. ATP also caused a drop of turbidity of the myosin B solution, the maximal drop reaching 50%.

7. The magnesium-activated apyrase activity and adenylate kinase activity were detected in the water-extract of crayfish muscle.

8. No adenylate deaminase activity was demonstrated in crayfish myosin B.

9. Crayfish myosin B had a true ATPase, activated by Ca. In the presence of 0.6 *M* KCl and 10 mM Ca at 30° C., the enzyme showed two pH optima, a higher one at 9.0 and a lower one at 6.0. In the presence of 0.6 *M* KCl at pH 7–8, EDTA maximally enhanced the ATPase action, Qp being higher than 3000. The activating effect of Ca was sensitive to the inhibiting action of Mg. The Michaelis constant was 5×10^{-5} *M* in the presence of 10 mM Ca and 1×10^{-3} *M* in the presence of 10 mM EDTA.

10. The ATPase action of crayfish myosin B was very sensitive to heavy metals, such as Cu or Zn. PCMB easily blocked the enzyme action. However, PCMB in dilute concentrations rather enhanced the Ca-activated ATPase activity.

Literature Cited

Bowen, W. J., and T. D. Kerwin, 1954. A study of the effects of ethylenediaminetetraacetic acid on myosin adenosinetriphosphatase. *J. Biol. Chem.*, **291**: 237–247.

Buchthal, F., A. Deutsch, G. G. Knappeis and A. Munch-Petersen, 1951. Further investigations on the phosphorus and nucleotide uptake of actomyosin. *Acta Physiol. Scand.*, **24**: 365–384.

Colowick, S. P., and H. M. Kalckar, 1943. The role of myokinase in transphosphorylations. 1. The enzymatic phosphorylation of hexoses by adenylpyrophosphate. *J. Biol. Chem.*, **148**: 117–126.

Edsall, J. T., and J. W. Mehl, 1940. The effect of denaturing agents on myosin. II. Viscosity and double refraction of flow. *J. Biol. Chem.*, **133**: 409–429.

Engelhardt, V. A., 1946. Adenosinetriphosphatase properties of myosin. *Adv. in Enzymol.*, **6**: 147–192.

GILMOUR, D., AND J. H. CALABY, 1953. Physical and enzymatic properties of actomyosins from the femoral and thoracic muscles of an insect. *Enzymologia,* **16**: 23–33.

HUMPHREY, G. F., 1948. The adenosinetriphosphatase activity of myosins from marine animals. *Physiol. Comp. et Oecol.,* **1**: 89–94.

KIELLEY, W. W., AND L. B. BRADLEY, 1956. The relationship between sulfydryl groups and the activation of myosin ATPase. *J. Biol. Chem.,* **218**: 653–659.

KITAGAWA, S., AND Y. TONOMURA, 1957. Contractile proteins from adductors of *Pecten.* III. Failure to detect adenylic acid deaminase activity. *J. Biochem. (Tokyo),* **44**: 317–320.

KOMINZ, D. R., F. SAAD AND K. LAKI, 1957. Vertebrate and invertebrate tropomyosins. *Nature,* **179**: 206–207.

LAKI, K., 1957. Composition of contractile muscle proteins *J. Cell. Comp. Physiol.,* (in press).

LOHMANN, K., 1935. Über die Aufspalung der Adenylpyrophosphorsäure und Argininphosphorsäure in Krebsmuskulatur. *Biochem. Zeitschr.,* **282**: 109–119.

LOHMANN, K., AND L. JENDRASSIK, 1926. Kolorimetrische Phosphorsäure-Bestimmungen im Muskelextrakt. *Biochem. Zeitschr.,* **178**: 419–426.

MARUYAMA, K., 1954. Studies on adenosinetriphosphatase from various insect muscles. *J. Fac. Sci. Tokyo Univ.,* IV, **7**: 231–270.

MARUYAMA, K., 1957a. Contractile proteins from adductors of *Meretrix* and *Cristaria. Annot. Zool. Jap.,* **30**: 63–66.

MARUYAMA, K., 1957b. Interaction of insect actomyosin with adenosine triphosphate. *J. Cell. Comp. Physiol.,* (in press).

MARUYAMA, K., 1957c. A further study of insect actomyosin. *Sci. Pap. Coll. Gen. Educ. Univ. Tokyo,* **7**: 213–241.

MARUYAMA, K., 1957d. Sea-anemone apyrase. I. Identification of the reaction products. *Enzymologia,* (in press).

MARUYAMA, K., AND Y. TONOMURA, 1957. Comparative studies of myosin B-ATP system. *J. Res. Inst. for Catalysis (Hokkaido Univ.),* **5**: 55–70.

MOMMAERTS, W. F. H. M., 1948. The reaction between actomyosin and adenosine triphosphate. *J. Gen. Physiol.,* **31**: 361–375.

MORIWAKI, K., 1956. Effects of azide on the ATP content of fish and toad embryos. M.S. Thesis. Univ. of Tokyo.

SARKAR, N. K., 1950. Observations on the glycerinated *Limulus* muscle. *Enzymologia,* **14**: 288.

SCHNEIDER, C., 1946. Phosphorous compounds in animal tissues. 3. A comparison of methods for the estimation of nucleic acids. *J. Biol. Chem.,* **164**: 747–751.

SNELLMAN, O., AND M. TENOW, 1954. A contractile element containing tropomyosin (actotropomyosin). *Biochim. et Biophys. Acta,* **13**: 199–207.

SZENT-GYÖRGYI, A., 1945. Studies on muscle. *Acta Physiol. Scand.,* **9**: Suppl. 25.

SZENT-GYÖRGYI, A., 1951. Chemistry of muscular contraction. Sec. Edition. Academic Press, Inc., New York.

TARVER, E., AND M. F. MORALES, 1951. The reaction between actomyosin and various nucleotides and phosphates, as followed by ultraviolet absorption. *J. Cell Comp. Physiol.,* **37**: 235–236.

TONOMURA, Y., 1956. Physicochemical study on the actomyosin-adenosine triphosphate system. *J. Res. Inst. for Catalysis (Hokkaido Univ.),* **4**: 87–108.

TONOMURA, Y., H. MATSUMIYA AND S. KITAGAWA, 1957. The effect of ethylenediaminetetraacetic acid on the interaction between actomyosin and adenosine triphosphate. *Biochim. et Biophys. Acta,* **24**: 568–576.

TONOMURA, Y., K. YAGI AND H. MATSUMIYA, 1956. Contractile proteins from adductors of *Pecten.* II. Interaction with adenosine triphosphate. *Arch. Biochem. Biophys.,* **64**: 466–479.

TSAO, T. C., P. H. TAN AND C. M. PENG, 1956. A comparative, physicochemical study of tropomyosins from different sources. *Scientia Sinica,* **5**: 91–111.

DE VILLAFRANCA, G. W., 1955. Adenosinetriphosphatase activity of squid mantle muscle (*Loligo pealii*). *Biol. Bull.,* **108**: 113–119.

WEBER, H. H., AND H. PORTZEHL, 1952. Muscle contraction and fibrous proteins. *Adv. Protein Chem.,* **7**: 161–252.

WEBER, H. H., AND H. PORTZEHL, 1954. The transference of the muscle energy in the contraction cycle. *Progress in Biophysics,* **4**: 60–111.

THE OCCURRENCE OF CHITIN IN THE LOPHOPHORATE PHYLA

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Knowledge of the occurrence of chitin among animals is imperfect and often contradictory. Zoologists have had the reprehensible habit of applying the name "chitin" to any hardened brown or yellow structure. Chitin is a definite chemical substance whose presence can be demonstrated only by chemical tests and the name should not be applied unless such tests have been performed. The chemical composition of chitin is discussed by Richards (1951).

The expression "lophophorate phyla" is adopted as a convenient collective term for the three phyla that possess a lophophore, namely, Phoronida, Ectoprocta, and Brachiopoda. As I regard the former two classes of Bryozoa, Entoprocta and Ectoprocta, as independent phyla, the name Bryozoa lapses. The three phyla in question are also called tentaculate phyla after Hatschek and by some zoologists are regarded as classes of a single phylum Tentaculata; but the term "tentaculate" appears unfortunate as many other groups are provided with tentacles. I do not consider the union of the three lophophorate groups into one phylum as desirable although close relationship between them is not to be doubted. During the writing of accounts of these three phyla, I became dissatisfied with the available information regarding the occurrence of chitin among them and decided to add to that information.

Fair information of the occurrence of chitin in animals appears in the articles of Wester (1910), Schulze (1924), and Kunike (1925), and in Richards' book (1951). Wester used the chitosan test but Schulze based his statements on the work of his student Kunike who used a method that has been severely criticized. Hence it appears impossible to place any great reliance on the information in the articles of Schulze and Kunike. A valuable and discriminating article on chitin in animals was contributed by Rudall (1955).

The method employed here is the chitosan-iodine color test as given by Campbell (1929). His directions are repeated in Richards (1951) who also gives a long discussion of this and other tests for chitin. Briefly, the material, submerged in potassium hydroxide saturated in distilled water at room temperature, is heated in closed tubes to 160° C. in a glycerin bath and held at this temperature for about 20 minutes. Both at Beaufort and in New York I found that the hydroxide would start to boil at 155° C. The material, alive or preserved, after removal if necessary of adherent foreign material, was washed in tap water followed by distilled water and then dried on filter paper until it no longer wet the paper. Air drying, tried at first, was abandoned when found to leave too much air in the material causing it to float in the alkali. Any residue after the heating in alkali may contain chitin. After washing out the alkali in tap and distilled water, the residue is flooded with the iodine solution whereupon if chitinous it turns brown. The iodine solution is then removed and weak sulphuric acid added whereupon if

chitinous the residue turns violet or purple or in case of a strong test deep purplish black. If chitinous the residue will also dissolve in weak acetic acid and a white precipitate will be thrown down from this by addition of weak sulphuric acid. For brevity, the brown-purple color changes will be referred to below as positive color test.

Phoronida

Phoronids are soft-bodied vermiform animals that live in a tube of their own secretion to which there are generally adherent sand or rock grains or other foreign material. Wester (1910) reported a negative test with *Phoronis psammophila* but did not state whether he referred to the animal or the tube, presumably the former. Kunike (1925) declared that the "integument" of *Phoronis,* species unspecified, is chitinous. What is meant by integument is not clear but if the surface layer of the body wall is indicated, then Kunike's result was erroneous.

1. *Phoronis pallida.* This is a small delicate species of which some specimens with and without tubes were kindly presented by Dr. Meredith Jones, who had obtained them by dredging in San Francisco Bay. The animals dissolved completely without trace in hot alkali but the delicate tubes remained and gave a strong positive color test.

2. *Phoronopsis viridis.* A large number of tubes containing the worms were generously sent by Colonel Lee Miles who collected them at Bodega Bay, California. The worm of a grayish green color inhabits tubes much longer than itself, usually several inches long. These are erect stiff tubes composed of a secreted parchment-like material covered with adherent rock grains. Piece of tubes and animals removed from the tubes were treated in hot alkali. As before the worms dissolved completely, coloring the alkali green. The tubes, from which most of the adherent grains had fallen off, remained and gave a very strong positive color test. The treated tubes also dissolved in weak acetic acid and a white precipitate appeared in the solution on addition of weak sulphuric acid.

The foregoing results show that the body of phoronids is devoid of chitin but their secreted tubes are composed of chitin.

Marine Ectoprocta

The ectoproct animal inhabits an exoskeletal case of its own secretion to the inner side of which part of its body wall is immovably fixed. The case is termed "zoecium" and the case plus the adherent body wall is called "cystid." The remainder of the animal, termed "polypide," is movable and can be partly extruded from the opening of the cystid (orifice) by a process of evagination. Upon disturbance the polypide is rapidly invaginated into the cystid and is so found in most preserved material.

Wester (1910) reported that the exoskeleton, which he called cuticle, is chitinous in *Zoobotryon verticillatum* (= *pellucidum*), *Flustra foliacea, Flustra carbasea,* and *Bugula turbinata.* The first of these is a ctenostome, the other three cheilostomes. Wester also mentioned a positive test in *"Therma tubulata."* It has proved impossible to ascertain what this is; there appears to be no such generic name as *Therma* in animals. Schulze (1924) listed Bryozoa as chitinous and this statement has been widely quoted but in fact Kunike (1925) did not test any marine

ectoprocts. Richards (1951) in his tabulation of the occurrence of chitin in animals incredibly mangled the available data on Bryozoa (page 45). He listed three categories: most Entoprocta, *Bugula, Barentsia* (misspelled); and stated that the part tested was the operculum. But none of these three categories has any operculum; although *Bugula* is a cheilostome it lacks an operculum. Of the forms indicated by Richards as entoprocts, only *Pedicellina* is such; and of course *Barentsia* is also an entoproct. The other forms listed as entoprocts are ectoprocts. Because of a lack of material I did not make any tests on entoprocts about which Wester reported that the cuticle of *Pedicellina* is partly chitinous whereas Kunike obtained a negative result with *Barentsia discreta*.

Work on marine ectoprocts was done during a sojourn in August, 1957, at the Duke University Marine Laboratory at Beaufort, North Carolina. It is a pleasure to acknowledge my indebtedness to Dr. Frank Maturo for collecting and identifying the material. The material was alive at the beginning of the test unless otherwise stated. After return to New York some additional species found preserved in the collections of the American Museum of Natural History were tested.

1. Ctenostomes. The ctenostomatous ectoprocts have membranous exoskeletal cases devoid of calcareous material, borne on running stolons clothed with the same exoskeletal secretion. A number of species were examined.

Amathia convoluta. Only the exoskeleton of zoids and stolons survived the treatment with hot alkali and gave a positive color test, also dissolving in weak acetic acid from which a precipitate was obtained with weak sulphuric acid.

Anguinella palmata. This forms bushy growths covered with a grayish green deposit. Part of this was removed by brushing and some fell off in the hot alkali. The underlying true exoskeleton of zoids and stolons gave a positive color test.

Zoobotryon verticillatum (preserved). The residue after the treatment with hot alkali gave an excellent positive color test, in confirmation of Wester's result, and dissolved in weak acetic acid.

Alcyonidium gelatinosum (preserved). This ctenostome occurs as gelatinous cylindroid growths composed of numerous contiguous zoids. The gelatinous material appears external to the layer corresponding to the exoskeleton of other ctenostomes. In the hot alkali much of the colonies dissolved, including the gelatinous material. The remaining material, appearing as outlines of the zoidal cases, gave a strong positive color test and dissolved in weak acetic acid.

Alcyonidium mytili (preserved). As in the preceding species outlines of the zoidal cases remained after the treatment with alkali, gave a strong positive color test, and also dissolved in weak acetic acid. In both species a white precipitate appeared in this solution on addition of weak sulphuric acid.

2. Cheilostomes. In the cheilostomatous ectoprocts the orifice of the exoskeletal case is displaced ventrally and is closeable by a little exoskeletal lid, the operculum. It happens, however, that an operculum is wanting in *Bugula* and some related genera. The exoskeletal case is generally thick and inflexible, even when devoid of calcareous deposition, and this condition is compensated by the occurrence of a thin ventral area of body wall, termed the "frontal membrane," whose outer layer is also exoskeletal. The first three species listed are devoid of calcareous deposition, the rest have a calcareous layer between the epidermis and the membranous layer of the exoskeleton. No attempt was made to remove the calcareous layer as it did not seem to interfere with the tests.

Aetea anguina. The minute solitary zoids of this species gave an excellent color test throughout the whole exoskeleton.

Bugula neritina. The entire residue from the alkali treatment gave an excellent positive color test but only partly dissolved in weak acetic acid.

Bugula turrita (preserved). The exoskeleton remained practically intact after the alkali treatment and gave an absolutely typical color test. It is impossible to understand the negative result of Richards and Cutkomp (1946) on *Bugula,* presumably this species. The residue also dissolved in weak acetic and the solution gave a precipitate with weak sulphuric.

Schizoporella unicornis. A positive color test was obtained from membranous material around the orifice and from the avicularia.

Thalamoporella gothica. This species fragmented greatly in the hot alkali and the residues gave a poor color test although they dissolved partly in weak acetic acid.

Membranipora tenuis. The zoids of the genus *Membranipora* have a calcareous back and the whole ventral surface consists of a frontal membrane. The membranes hanging to the residues, including the operculum, gave a good positive color test.

Membranipora tuberculata. This gave the same result as the preceding species. The frontal membranes gave an excellent color test and dissolved in acetic acid.

Conopeum commensale. The frontal membranes including the little spines borne on them in this genus and the surface membranes everywhere gave an excellent positive color test.

Hippothoa hyalina. The opercula and some other surface parts were positive in the color tests.

Parasmittina trispinosa. A positive color test was obtained from the avicularia, small areas around the orifice, and the pores of the ovicells.

3. Cyclostomes. The cyclostomatous ectoprocts are heavily calcified, having tubular calcareous cases with terminal orifice and no operculum. Presumably the calcareous material is covered with a cuticle. Only minute fragments of one species were available.

Crisia eburnea. The color test was negative. In the genus *Crisia* the branches are jointed and these joints consist of thickened cuticle without calcareous deposition. These joints dissolved in the hot alkali, hence are not chitinous.

The foregoing results demonstrate that in ctenostomes and non-calcareous cheilostomes the entire exoskeleton consists of chitin. In calcareous cheilostomes, the avicularia, the opercula, the frontal membranes, and some other parts are generally chitinous. The one cyclostome tested gave a negative result but other cyclostomes should be tried.

Fresh-Water Ectoprocts

Fresh-water ectoprocts have a wholly membranous exoskeleton. Only preserved material was available and most of this was kindly furnished by Dr. Mary Rogick. Zander (1897) reported a negative result in *Plumatella* but Kunike (1925) claimed that the "integument" is chitinous in *Plumatella* and *Cristatella.* The jelly of *Pectinatella* contains chitin (Kraepelin, 1887; Morse, 1930). Lerner (1954) found that the float of the statoblasts of *Plumatella* and *Cristatella* is insoluble in alkali and concluded that it is chitinous.

1. Phylactolaemates. This group includes the typical fresh-water ectoprocts with horseshoe lophophore.

Plumatella emarginata. The exoskeleton survived treatment and gave an excellent positive color test, also dissolving in weak acetic acid.

Plumatella repens. A colony with well expanded polypides was found in the collections of the American Museum of Natural History. The polypides dissolved completely in the hot alkali leaving the exoskeletal tubes and contained statoblasts and both were very positive in the color tests.

Fredericella sultana. This species behaved like *Plumatella.* The entire exoskeleton survived the alkali and was highly positive in the color tests, also dissolving in weak acetic acid.

Pectinatella magnifica, statoblasts. Of course the germinal mass dissolved in alkali but the float remained although losing its brown color. Traces of the hooks were left but these disintegrated on washing in water. The float was highly positive.

2. Gymnolaemates. There are some relatives of marine ectoprocts found in fresh water, known by the circular lophophore.

Paludicella articulata. The small colonies were much disrupted by the treatment with alkali but the fragments gave a good color test.

From the foregoing results it is evident that the entire exoskeleton of fresh-water ectoprocts consists of chitin and the same is true of the float of the statoblasts.

Brachiopoda

All materials except *Crania* had been preserved in fluid. *Lingula* was purchased from a biological supply house but the other preserved species were kindly supplied by Dr. Francis Stehli.

1. Inarticulates. It is generally recognized that the shells of inarticulate brachiopods except the Craniidae contain a large amount of chitin. This statement rests on positive tests for chitin in *Lingula* by Schmiedeberg (1882), Krükenberg (1885), and Wester (1910). Wester states that the shell of *Lingula* contains a great deal of chitin and that the thick cuticle of the pedicle is also chitinous. Rudall (1955) records that the mantle setae and the cuticle of the pedicle of *Lingula* are "very well-defined examples of beta chitin."

Lingula. In confirmation of previous results pieces of the shell and the thick cuticle of the pedicle surviving the treatment with hot alkali gave a very strong positive color test. The interior tissues of the pedicle, consisting of connective tissue and muscle, dissolved in the hot alkali. The cuticle after the treatment dissolved in weak acetic acid and the solution threw down a white precipitate on addition of weak sulphuric acid. The setae were not recovered.

Discinisca lamellosa. Pieces of shell surviving the hot alkali colored poorly in the iodine solution but gave a strong dark purple response to sulphuric acid. When placed in acetic acid these pieces did not dissolve noticeably but a white precipitate appeared in the solution on addition of weak sulphuric acid. The membrane covering the slot in the ventral valve is also chitinous. Discinisicids lack a pedicle. The mantle setae were not recovered. The apex of the dorsal valve appeared devoid of chitin.

Crania anomala (dry). Although pieces of the shell after the alkali treatment

turned slowly brown in iodine they gave no change of color in weak sulphuric acid and did not dissolve in acetic acid. This confirms the accepted opinion that the shell of the Craniidae lacks chitin. The Craniidae have no pedicle.

Chitin is therefore abundantly present in the shell of inarticulate brachiopods except Craniidae and the mantle setae and cuticle of the pedicle are also chitinous.

2. Articulates. The shell of articulates is generally regarded as calcareous without any chitinous component. Wester (1910) obtained a negative result for chitin with an unidentified articulate. Kunike (1925) likewise reported a negative test in *"Terebratula"* (an extinct genus).

Terebratulina retusa (= *caputserpentis*). A negative color test was obtained with the shell of this species.

Terebratalia transversa. Pieces of the shell after the alkali treatment were negative for chitin but fragments of the short pedicle of this form were strongly positive.

Laqueus californianus. This species stands out from the substrate by a neat pedicle of some length. Although the pieces of shell after the alkali treatment were negative for chitin, the surviving outer layer of the pedicle was strongly positive.

It is clear from the available data that the valves of articulate brachiopods are devoid of chitin but the cuticle of the pedicle is chitinous as in lingulids.

Conclusion

The distribution of chitin among animals is probably not of phylogenetic significance. Nevertheless it is suggestive that chitin is the common exoskeletal secretion in the three, closely related, lophophorate phyla whereas it appears entirely wanting in another assemblage of related phyla, the deuterostomes (Echinodermata, Hemichordata, Chordata). The total lack of chitin in echinoderms and chordates is well established. In regard to hemichordates no test appears to have been made on enteropneusts but Rudall (1955) testifies that the coenecium of *Rhabdopleura* disintegrates rapidly in hot alkali and gave no evidence of chitin on x-ray examination. The Pogonophora no doubt belong among the deuterostomes and it would be interesting to make a test on their tubes.[1] Whether chaetognaths can be included in the deuterostomes is indeterminable on present evidence, but in this connection it is suggestive to note that their grasping spines are made of chitin. Although Schmidt (1940) obtained a negative result on these spines with the Schulze-Kunike method, I found that they are highly positive by the chitosan test. Preserved specimens of *Sagitta elegans* dissolved completely in the hot alkali except for the grasping spines which gave a strong color reaction. This finding possibly indicates some divergence of chaetognaths from typical deuterostomes.

[1] Since the above was written, Professor A. V. Ivanov, of the Academy of Sciences of Leningrad, has kindly informed that the tubes of the Pogonophora have been tested chemically and found to consist of cellulose.

LITERATURE CITED

Campbell, F. L., 1929. The detection and estimation of insect chitin, and the irrelation of chitinization to hardness and pigmentation of the cuticula of the American cockroach, *Periplaneta americana* L. *Ann. Entomol. Soc. America,* **22**: 401–426.

KRAEPELIN, K., 1887. Die deutschen Süsswasserbryozoen. I. Anatomischer-systematischer Teil. *Abhandl. Gebiete Naturwiss. Naturwiss. Verein Hamburg*, 10, no. 9, 168 pp.

KRÜKENBERG, C. F. W., 1885. Über das Vorkommen des Chitins. *Zool. Anz.*, **8**: 412–415.

KUNIKE, G., 1925. Nachweis und Verbreitung organischer Skeletsubstanzen bei Tieren. *Zeitschr. vergl. Physiol.*, **2**: 233–253.

LERNER, HEDWIG, 1954. Zur Kenntnis des Feinbaues der Flottoblastenschalen von *Plumatella repens* (L.) und *Cristatella mucedo* Cuvier. *Ber. Oberhess. Gesell. Natur- und Heilk. Giessen, Naturiss. Abt.*, New ser. **27**: 111–122.

MORSE, W., 1930. Chemical constitution of *Pectinatella*. *Science*, **71**: 265.

RICHARDS, A. GLENN, 1951. The Integument of Arthropods. xvi + 411 pp., University of Minnesota Press, Minneapolis.

RICHARDS, A. G., AND L. K. CUTKOMB, 1946. Correlation between the possession of a chitinous cuticle and sensitivity to DDT. *Biol. Bull.*, **90**: 97–108.

RUDALL, K. M., 1955. The distribution of collagen and chitin. *In*: R. Brown and J. F. Danielli (eds.), Fibrous proteins and their biological significance. *Symposia Soc. Exptl. Biol.*, no. **IX**: 49–71.

SCHMIDT, W. J., 1940. Zur Morphologie, Polarisationsoptik, und Chemie der Greifhaken von *Sagitta hexaptera*. *Zeitschr. Morphol. Ökol. Tiere*, **37**: 63–82.

SCHMIEDEBERG, O., 1882. Ueber die chemische Zusammensetzung der Wohnröhren von *Onuphis tubicola* Müll. *Mitt. Zool. Stat. Neapel*, **3**: 373–392.

SCHULZE, P., 1924. Der Nachweis und die Verbreitung des Chitins mit einem Anhang über das komplizierte Verdauungssystem der Ophyroscoleciden. *Zeitschr. Morphol. Ökol. Tiere*, **2**: 643–666.

WESTER, D. H., 1910. Über die Verbreitung und Lokalization des Chitins im Tierreiche. *Zool. Jahrb. Abt. System.*, **28**: 531–558.

ZANDER, E., 1897. Vergleichende und kritische Untersuchungen zum Verständnisse der Iodreaktion des Chitins. *Arch. Ges. Physiol.*, **66**: 545–573.